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PRINCIPAL INVESTIGATOR: Yuichi Machida, Ph.D.
Anindya Dutta, M.D., Ph.D.

CONTRACTING ORGANIZATION: Brigham and Women's Hospital
Boston, Massachusetts 02115

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6. AUTHOR(S) :
Yuichi Machida, Ph.D.
Anindya Dutta, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Brigham and Women's Hospital
Boston, MA 02115

E-Mail:ymachida@rics.bwh.Harvard.edu

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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
Recent studies show that 80-90% of breast carcinomas are associated with gene amplification. Each amplicon most likely has an origin of replication. In Drosophila, DmORC (Origin Recognition Complex) binds to specific DNA sequences and this binding is essential for chorion gene amplification. To study the implications of replication initiator proteins to gene amplification, we characterized two proteins required for DNA replication (ORC2 and Mcm10). We created hypomorphic mutation in the ORC2 gene of a cancer cell line through homologous recombination. We found that these cells failed to support the replication of an extrachromosomal plasmid bearing the oriP replicator of Epstein Barr virus (EBV). Geminin, an inhibitor of replication initiation complex, inhibited replication of this episome. The result identifies a novel means by which to cure cancer with gene amplification where the amplicons are carried as episomes. We also report that Xenopus Mcm10 (XMcm10) is not required for origin binding of XMcm2-7. Instead, the chromatin binding of XMcm10 at the onset of DNA replication requires chromatin-bound XMcm2-7, and it is independent of Cdk2 and Cdc7. In the absence of XMcm10, XCdc45 binding, XRPA binding, and initiation-dependent plasmid supercoiling are blocked. Therefore, XMcm10 performs its function after pre-RC assembly and before origin unwinding.

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Introduction

Recent studies show that 80-90% of breast carcinomas are associated with gene amplification (1, 2). Unfortunately, no effort has been put so far to study the mechanism of gene amplification from a view of DNA replication. Origins of replication are first recognized and bound by a six-subunit origin recognition complex (ORC), which was first identified in yeast. In Drosophila, DmORC (Origin Recognition Complex) binds to specific DNA sequences and this binding is essential for chorion gene amplification. One certain way to diminish the amount of amplification is to inhibit the initiation of replication in the double minute chromosome. To study the implications of replication initiator proteins to gene amplification, we characterized two proteins required for DNA replication (ORC2 and Mcm10). We also report that inhibition of DNA replication initiation by geminin diminishes extra-chromosomal DNA replication.

Results

Geminin Inhibits Replication of episome

To investigate the function of ORC in gene amplification in human cells, we created a hypomorphic mutation in the ORC2 gene of a cancer cell line through homologous recombination. This mutation reduced ORC2 level by 90%. The G1 phase of the cell cycle was prolonged, but there was no effect on the utilization of either the c-myc and β-globin cellular origins of replication. However, we found that cells carrying this mutation failed to support the replication of an extrachromosomal plasmid bearing the oriP replicator of Epstein Barr virus (EBV), and this defect was rescued by reintroduction of ORC2. ORC2 specifically associates with oriP in cells, most likely through its interaction with EBNA-1, an EBV-encoded protein which recognizes oriP sequence. The fact that ORC is required for replication from oriP suggests that other components of the replication initiation complex are also required.

We took advantage of this system to check whether inhibition of DNA replication diminish gene amplification where amplicons are carried as episomes. Geminin, a regulator of DNA replication, interacts with one of these downstream components, Cdt1, to prevent the loading of MCM proteins (3). Geminin is degraded by the anaphase-promoting complex and can be stabilized by mutation of a specific destruction box (gemininΔDB) (4). p367 (a plasmid containing oriP and EBNA1) was cotransfected into HCT116 cells with plasmid pEBG expressing glutathione S transferase (GST) as a control or with plasmids expressing GST-geminin or GST-gemininΔDB. Judging from the amount of DpnI resistant replicated plasmid DNA, we found that GST-geminin inhibits replication of p367. GST-gemininΔDB, which expresses a higher concentration of the protein, inhibits replication from p367 to a greater extent.

Mcm10 Is Required for DNA Replication

Some of the replication initiation factors identified in yeast have not been characterized in higher eukaryotes. One such factor is Mcm10. Mcm10 was first identified in S. cerevisiae as a gene required for chromosomal DNA replication and stable plasmid maintenance(5, 6). To determine whether Mcm10 is required for DNA replication in higher eukaryotes, we utilized in vitro replication system using Xenopus egg extracts. Extracts depleted of XMcm10 exhibited a 8-fold decrease in DNA replication compared to mock-depleted extracts, and this defect was completely reversed by addition of recombinant GST-XMcm10. Therefore, the replication defect

in XMcm10-depleted extracts was due to the selective removal of XMcm10. These data show that XMcm10 is essential for DNA replication in *Xenopus* extracts.

Chromatin Association of XMcm10 Requires a Functional Pre-RC but Is Independent of Cdc7 Activity

In the G1 phase of the cell cycle, Cdc6, Cdt1 and MCM2-7 complex are recruited to replication origins to form a prerepricative complex (pre-RC) (7). Given the genetic and physical interaction of yeast Mcm10 with other members of the yeast Mcm2-7 complex (5), we tested whether the XMcm2-7 complex is required for the chromatin loading of XMcm10. We used antibodies against XMcm7 to deplete the XMcm2-7 complex from egg cytosol and then examined the chromatin association of XMcm10 after addition of NPE. XMcm2-7-depleted extracts were unable to recruit XMcm10 to the chromatin. Similarly, the addition to egg cytosol of geminin, which blocks XMcm2-7 loading through its association with Cdt1, also prevented the recruitment of XMcm10 to replication origins. Together, these data suggest that the presence on chromatin of the XMcm2-7 complex is required for the recruitment of XMcm10 to replication origins.

Upon entry into S phase, DNA replication is initiated by the conversion of pre-RCs into active replication forks. This transformation requires the activity of two families of protein kinases, the Cdc7/Dbf4 kinase and the S phase cyclin-dependent kinases (Cdk), which cooperate to recruit Cdc45 to origins of DNA replication (7). We next determined whether XCdc7 was required for chromatin binding of XMcm10. We find that XMcm10 was efficiently loaded onto chromatin in the absence of XCdc7. We found that the addition of the Cdk2 inhibitor $p27^{Kip}$ did not inhibit chromatin binding by XMcm10. Therefore, recruitment of XMcm10 to replication origins is independent of both Cdk2 and Cdc7, but requires the XMcm2-7 complex.

XMcm10 Is Required for Chromatin Binding of XCdc45 and Origin Unwinding, but Not for XMcm2-7 Complex Recruitment

To determine at what step of replication initiation XMcm10 performs its function, we carried out chromatin binding experiments in XMcm10-depleted extracts in the presence of aphidicolin, which arrest system after initiation had occurred. Under these conditions, normal levels of XMcm7 bound to chromatin, but XCdc45 and XRPA binding in NPE was severely reduced, and the reduced binding was reversed by the addition of recombinant XMcm10. In this experiment, depletion of XMcm10 caused an ~6-fold reduction in DNA replication that was fully rescued by GST-XMcm10, and the overall efficiency of DNA replication was ~100%. We also examined the binding of XCdc7 to chromatin in XMcm10-depleted extracts. We found a small but reproducible decrease in the amount of chromatin-bound XCdc7 in XMcm10-depleted extracts. Thus, although our data show that XCdc7 loads onto chromatin independently of XMcm10, we cannot rule out the possibility that there is a pool of XCdc7 whose chromatin association is XMcm10 dependent. Together, these experiments show that XMcm10 is not required for pre-RC assembly, but rather for the recruitment of XCdc45 and RPA to origins of replication.

The lack of XCdc45 and XRPA binding in XMcm10-depleted extracts suggests that XMcm10 is required for origin unwinding. To test this directly, a circular plasmid was incubated in egg cytosol followed by NPE containing aphidicolin, and its topology was analyzed by DNA topology assay. In XMcm10-depleted extracts, origin unwinding is blocked at an early step. The

results of the chromatin binding and plasmid supercoiling assays therefore both show a requirement for XMcm10 at an early stage of origin unwinding.

Key Research Accomplishments

- Geminin inhibits replication of episome
- XMcm10 is required for DNA replication
- Chromatin association of XMcm10 requires a functional pre-RC but is independent of Cdc7 activity
- XMcm10 is required for chromatin binding of XCdc45 and origin unwinding, but not for XMcm2-7 complex recruitment

Reportable Outcomes

Publications

1. Dhar SK, Delmolino L, Dutta A. (2001) Architecture of the human origin recognition complex. *J Biol Chem* **276**:29067-29071.
2. Dhar SK, Yoshida K, Machida Y, Khaira P, Chaudhuri B, Wohlschlegel JA, Leffak M, Yates J, Dutta A. (2001) Replication from oriP of Epstein-Barr virus requires human ORC and is inhibited by geminin. *Cell* **106**:287-296.
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Abstracts

Dhar SK, Yoshida K, Machida Y, Hwang D-S, and Dutta A. (2001) Genetic evidence for the role of human ORC in DNA replication. Cold Spring Harbor Laboratory Meeting.

Cell line

HCT116 containing hypomorphic mutation in *Orc2* gene (Δ/-)

Conclusions

In this study, we found that geminin inhibits replication of episome in human cells. Since episomes contain only one origin of replication while chromosomes contain multiple origins (not all of which are necessary for cell survival), it is likely that episomes would be far more sensitive to geminin-based drugs that specifically target initiation. This idea is supported by the fact that overexpression of geminin has only a minor effect on cell cycle progression of mammalian cells (data not shown), whereas episomal replication is strongly inhibited. The result concurrently identifies a novel means by which to cure cancer with gene amplification where the amplicons are carried as episomes. We would check whether formation of double minutes is inhibited by geminin. We would also test whether ORC2 is involved in gene amplification by using ORC2 (Δ/-) cell line. RNAi targeting replication initiation protein will be used to test whether other replication initiation proteins including Mcm10 is involved in gene amplification.

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Architecture of the Human Origin Recognition Complex*

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Suman Kumar Dhar‡, Laurie Delmolino§, and Anindya Dutta¶

From the Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

All the human homologs of the six subunits of *Saccharomyces cerevisiae* origin recognition complex have been reported so far. However, not much has been reported on the nature and the characteristics of the human origin recognition complex. In an attempt to purify recombinant human ORC from insect cells infected with baculoviruses expressing HsORC subunits, we found that human ORC2, -3, -4, and -5 form a core complex. HsORC1 and HsORC6 subunits did not enter into this core complex, suggesting that the interaction of these two subunits with the core ORC2–5 complex is extremely labile. We found that the C-terminal region of ORC2 interacts directly with the N-terminal region of ORC3. The C-terminal region of ORC3 was, however, necessary to bring ORC4 and ORC5 into the core complex. A fragment containing the N-terminal 200 residues of ORC3 (ORC3N) competitively inhibited the ORC2-ORC3 interaction. Overexpression of this fragment in U2OS cells blocked the cells in G_1, providing the first evidence that a mammalian ORC subunit is important for the G_1-S transition in mammalian cells.

Origin recognition complex (ORC)[1] was first described in yeast *Saccharomyces cerevisiae* (1). All six subunits, essential for cell viability, collectively bind to the ARS (autonomously replicating sequence) consensus sequence in a sequence-specific manner and lead to the chromatin loading of other replication factors like CDC6 and MCM (mini-chromosome maintenance) that are essential for initiation of DNA replication (2, 3). Similar six protein complexes have been discovered in *Xenopus laevis* (4), *Drosophila melanogaster* (5) and *Schizosaccharomyces pombe* (6), although a consensus DNA sequence that serves as an origin of replication and where ORC may bind has not been found in these species. Conservation of similar ORC subunits in mammals suggests that ORC has an equally important role in mammalian cells.

Although all six human homologs of yeast *S. cerevisiae* ORC subunits have been reported (7–14), purification of a six-protein human origin recognition complex remains elusive. Endogenous ORC2, -3, -4, and -5 subunits have been reported to interact with each other in extracts of cancer cell lines (14). ORC1 and ORC6 did not interact with other ORC subunits under these experimental conditions (14). Therefore it is possible that a functional human ORC exists only during a very short period of the cell cycle or in a specific sub-nuclear compartment, making it difficult to extract such a complex from human cell lines. In fact, in a recent study hamster ORC1 was reported to be easily eluted from chromatin during mitosis and early G_1 phase (15). It became stably bound to chromatin again during mid-G_1 phase with the appearance of a functional pre-replication complex at a hamster replication origin. In contrast, ORC2 was stably bound to chromatin throughout the cell cycle. Difficulties in obtaining six protein human ORCs may also be attributed to the fact that we are still missing some of the unidentified important components of the human ORC. Indeed, immunoprecipitation from [^{35}S]methionine-labeled HeLa cell lysate of ORC1, -2, -3, -4, and -6 showed many non-ORC proteins interacting specifically with the respective ORC subunits (14, 16).

A six protein ORC has been purified from *Drosophila* embryo extracts and possesses some demonstrated biochemical activities (5, 17, 18). All six *Drosophila* ORC subunits were expressed and subsequently purified to homogeneity from baculovirus-infected insect cells (17). Using an *in vitro* transcription translation reaction, a similar six-protein ORC has been reported in yeast *S. pombe* (6). With all the six human ORC subunits in our hand, we attempted to produce recombinant human ORC from the baculovirus expression system in order to dissect the activities and architecture of human origin recognition complex/subcomplex(s).

Because genetic experiments are difficult to perform in mammalian systems, the human ORC subunits have not been shown to have a role in replication or cell proliferation. Utilizing knowledge learned about the architecture of the human ORC, we created a dominant negative ORC subunit designed to disrupt the formation of endogenous ORC. Overexpression of this dominant negative ORC subunit blocked the cell cycle in G_1, providing the first evidence of the importance of ORC in cancer cell proliferation.

MATERIALS AND METHODS

Plasmid Constructions—Cloning of ORC1–6 cDNAs are described elsewhere (7, 9–11, 14). Coding sequences of ORC1, ORC4, ORC5, ORC6, and ORC3N200 were cloned into pFastBac (Life Technologies, Inc.), and coding sequences of ORC2 and ORC3 were cloned into pFastBac Dual. ORC2, -4, and -5 were also cloned in pFB-GST vectors to express GST fusion proteins. ORC3 and all the related constructs for *in vitro* transcription and translation reactions were made into T7T3DPAC vector (GenBank™ accession number U13871). Full-length and C-terminal ORC2 fragments were cloned into pGEX-5X-3 (Invitrogen) to produce bacterial fusion proteins. Additional information regarding the constructs will be made available upon request.

Expression of ORC Subunits in Insect Cells, Purification, and Gel Filtration—Baculoviruses were produced from the recombinant pFastBac or pFB-GST plasmids using the Bac-to-Bac expression system (Life Technologies, Inc.). Hi5 or Sf9 cells (Invitrogen) were infected with

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¶ To whom correspondence should be addressed: Dept. of Pathology, Brigham and Women's Hospital, Harvard Medical School, 75 Francis St., Boston MA 02115. Tel.: 617-278-0468; Fax: 617-732-7449; E-mail: adutta@rics.bwh.harvard.edu.

[1] The abbreviations used are: ORC, origin recognition complex; GST, glutathione S-transferase; FACS, fluorescence-activated cell sorter; GFP green fluorescent protein.

these baculoviruses according to the manufacturers' recommendations. Cells were harvested 48 h post-infection. The cell pellet was washed once in cold phosphate-buffered saline and subsequently resuspended in hypotonic lysis buffer (10 mM Tris/Cl, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$,1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM dithiothreitol). The cell suspension was homogenized in a Dounce homogenizer using a B-type pestle followed by centrifugation at 3000 rpm for 7 min. The pellet containing the nuclei was lysed in buffer H/0.15 (50 mM HEPES/KOH, pH 7.5, 150 mM KCl, 0.02% Nonidet P-40, 5 mM magnesium acetate, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM dithiothreitol). The resulting suspension was subjected to ammonium sulfate precipitation (starting with 10% followed by 30% and finally 50%). The pellet after the 50% ammonium sulfate cut was resuspended in buffer H/0.0 (no salt) and then dialyzed overnight against buffer H/0.15. The dialyzed sample was then bound to GST beads (Sigma) and washed three times with buffer H/0.25. Proteins were eluted using reduced glutathione elution buffer (50 mM Tris/Cl, pH 8.0, 20 mM reduced glutathione, 0.01% Nonidet P-40, 100 mM NaCl). Gel filtration of glutathione eluate using a fast protein liquid chromatography Superose 12 (Amersham Pharmacia Biotech) column was performed as described previously (14).

Cell Culture, Transfection, Immunoblotting, Immunoprecipitation, and Silver Stain—Sf9 and Hi5 cells were maintained according to the manufacturers' protocol (Invitrogen). U2OS cells used for FACS analysis were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Life Technologies, Inc.). Plasmid DNA used for transfection were purified using Qiagen maxiprep kits. Cells were grown in 100-mm dishes and transfected using LipofectAMINE (Life Technologies, Inc.). Western blotting and immunoprecipitation techniques were carried out using standard protocols. Anti-GST polyclonal antibodies were purchased from Santa Cruz Biotechnologies. Antibodies against HsORC1–6 have been described previously (9–11, 14). The silver stain method has been described elsewhere (19).

In Vitro Transcription and Translation Reactions and GST Pull-down Assay—*In vitro* transcription and translation reactions to produce [^{35}S]methionine-labeled full-length and different deletions of ORC3 were performed using the Promega TNT system (Madison, WI). Pull-down assays on glutathione-agarose beads were done as described previously (20).

FACS Analysis—U2OS cells were transfected with farnesylated GFP (CLONTECH) alone or in combination with FLAGORC2, GFPC1-ORC3N, or GFPC1-ORC3C1. Forty-eight hours after transfection, cells were trypsinized, washed with phosphate-buffered saline, fixed with cold 70% ethanol, and stored until further use. Before analysis, fixed cells were resuspended in phosphate-buffered saline containing 50 μg/ml propidium iodide (Sigma), 10 μg/ml RNaseA (Sigma), and 0.05% Noniodet P-40 and then incubated for 1 h at 4 °C. Finally cells were washed in phosphate-buffered saline and analyzed by flow cytometry. The data were further analyzed using FLOWJO software to calculate the percentage of cells residing in different cell cycle stages.

RESULTS

GSTORC5, -2, -3, and -4 Forms a Complex—ORC2, -3, -4, and -5 subunits have been shown to interact with each other in human cell extracts (14). In an attempt to purify recombinant six protein human origin recognition complex, we infected Sf9 insect cells with baculoviruses expressing human ORC1–6 subunits. One of the subunits, ORC5, was GST-tagged. After pull down on glutathione beads, we found that GSTORC5, -2, -3, and -4 can be purified as a complex (Fig. 1*A*). ORC1 did not enter into the complex in a stoichiometric ratio, and the presence of very little ORC1 in Fig. 1*A* was not reproducible in different preparations. ORC6 did not enter into the complex at all. Both ORC1 and ORC6 were expressed at a high level. In a control experiment, we expressed GST alone with other ORC subunits. Pull down on glutathione beads purified only GST but none of the ORC subunits. Therefore, the results in Fig. 1*A* are due to the formation of a complex of ORC2, -3, -4, and -5 and not due to precipitation of the proteins on the glutathione beads. GST pull-down experiments using GST tags on different ORC subunits (GSTORC2 and GSTORC4) confirmed the previous result showing ORC2, -3, -4, and -5 form a core complex (data not shown).

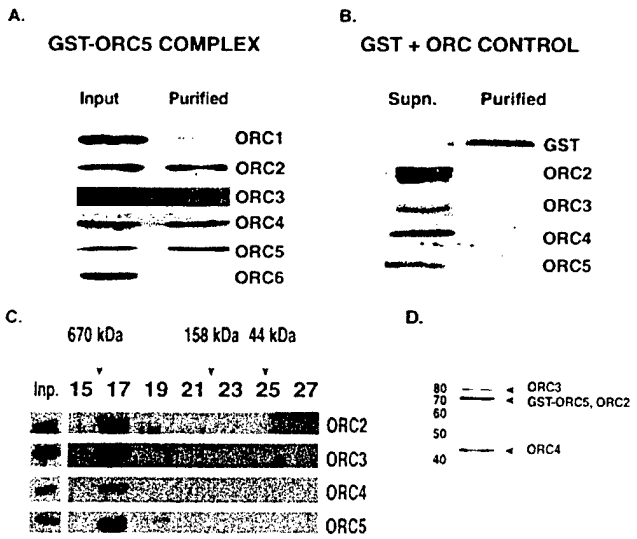

FIG. 1. **Human ORC2–5 co-purify in a complex.** *A*, Sf9 insect cells were infected with baculoviruses expressing ORC1, -2, -3, -4, -6 and GSTORC5. Proteins were purified as described under "Materials and Methods." Both purified proteins and crude lysate (*Input*) were immunoblotted using ORC1–6 antibodies. *B*, Sf9 insect cells were infected the same way as in *A* using baculoviruses expressing ORC2–5 and GST as control. The cell lysate was purified on GST beads and immunoblotted. *C*, Sf9 insect cells were infected with baculoviruses expressing ORC2, -3, -4 and GST-ORC5. Proteins purified on GST beads were fractionated on a Superose 12 gel filtration column. Alternate fractions were immunoblotted using anti-ORC2, -3, -4 and anti-GST antibodies. The positions of the molecular mass markers thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), and chicken ovalbumin (44 kDa) are shown on top. Input *lanes* were loaded with 5% of the total lysate passed through the column.

To further show that GSTORC5, -2, -3, and -4 subunits are in one complex, we analyzed the elution pattern of these proteins upon gel filtration. Proteins were eluted from the GST beads using reduced glutathione and subsequently passed through a Superose 12 gel filtration column. Upon Western blotting of different fractions with different anti-ORC antibodies, we found that GSTORC5, -2, -3, and -4 subunits were co-eluted in one fraction (Fig. 1*C*). The molecular mass of this complex is ~500 kDa, which is more than the combined molecular mass of the four ORC subunits. This may be because of the multimerization of the GST moieties to give a high molecular mass complex or because the complex has an atypical shape. Silver staining of the purified protein used for the gel filtration experiment indicated that GST-ORC5, ORC2, -3, and -4 were the only proteins present in the preparation in significant amounts (Fig. 1*D*).

ORC2 and ORC3 Physically Interact with Each Other—We were interested in seeing which of the four interacting subunits interact directly. Sf9 insect cells were infected with six different combinations of baculoviruses expressing two ORC subunits in each case. One of the two viruses was GST-tagged. Affinity purification on glutathione beads showed that only ORC2 and ORC3 directly interacted with each other (Fig. 2). None of the other dual combinations of baculovirus showed any interaction under our experimental conditions. Therefore, we conclude that ORC2 and ORC3 form a core component of the ORC2, -3, -4, -5 complex.

ORC2 and ORC3 Recruit ORC4 and -5—The ORC2-3 complex is expected to recruit ORC4 and ORC5. We were interested in seeing whether ORC2-3 core complex can recruit ORC4 first, followed by ORC5, or vice versa. Sf9 cells were infected by baculoviruses expressing GSTORC4, -2, and -3 subunits or by viruses expressing GSTORC5, -2, and -3. GSTORC4 did not

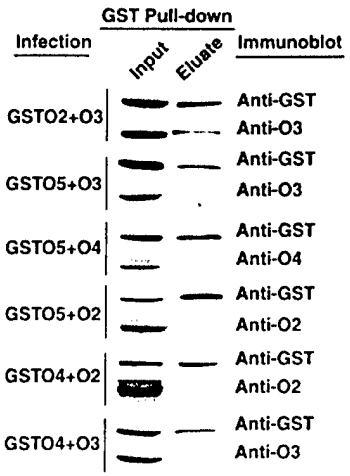

FIG. 2. **Direct interaction between ORC2 and ORC3 subunits.** Sf9 insect cells were infected with six different combinations of baculoviruses expressing two ORC subunits in each case (GSTORC2-3 (*GSTO2+03*), GSTORC5-3 (*GSTO5+03*), GSTORC5-4 (*GSTO5+04*), GSTORC5-2 (*GSTO5+02*), GSTORC4-2 (*GSTO4+02*), and GSTORC4-3 (*GSTO4+03*)). Proteins bound to GST beads were immunoblotted using either anti-GST antibody or respective anti-ORC antibodies. 5% of the total lysate was loaded in the input *lanes*.

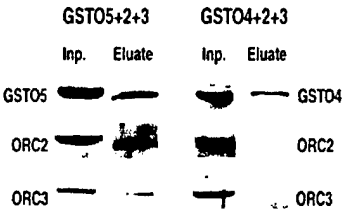

FIG. 3. **ORC2-3 subcomplex can load ORC5 but not ORC4.** Sf9 insect cells were infected either with baculoviruses expressing GSTORC5-2-3 (*GSTORC5+2+3*) or baculoviruses expressing GSTORC4-2-3 (*GSTORC4+2+3*). Proteins bound to GST beads were immunoblotted using either anti-GST antibodies or respective anti-ORC antibodies. Input (*Inp.*) *lane* contains 5% of proteins input on GST beads.

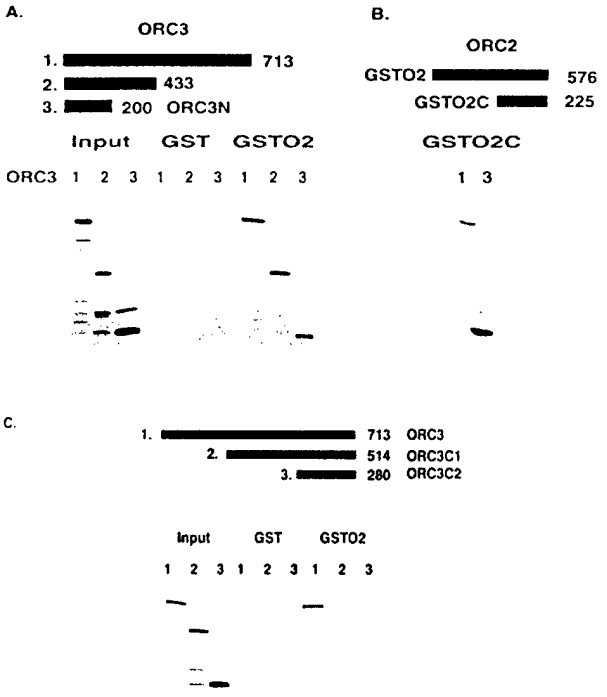

FIG. 4. **Mapping domains of interactions between ORC2 and ORC3.** *A*, full-length ORC3 or different N-terminal deletions of ORC3 were produced using *in vitro* transcription and translation reactions and tested for their ability to bind either GST or GSTORC2 in a pull-down experiment on glutathione-agarose beads coated with GST, GSTORC2 (*GSTO2*). In each case, the input *lanes* were loaded with 5% of the amount of the labeled protein incubated with the beads. The labeled proteins were visualized by SDS-polyacrylamide gel electrophoresis followed by fluorography. *B*, GST pull-down experiment as in *A* using GSTORC2C (C-terminal portion of ORC2) and *in vitro* transcribed and translated full-length ORC3 (*1*) or ORC3N200 (*3*). *C*, full-length ORC3 or different C-terminal deletions of ORC3 were produced using *in vitro* transcription translation reaction. GST pull-down experiments were performed as shown in Fig. 5*A* using either GST or GSTORC2.

interact with ORC2 and ORC3, whereas GSTORC5 interacted with ORC2 and ORC3 (Fig. 3). Therefore, ORC2-3 core complex is capable of recruiting ORC5, but it cannot recruit ORC4 by itself. The fact that ORC2, -3, -4, and -5 form a complex suggests that ORC2, -3, and -5 complex is necessary to load ORC4. It is also possible that ORC4 and ORC5 can be loaded on ORC2-3 core complex simultaneously independent of each other, but ORC5 is necessary to stabilize the association of ORC4 with the other ORC subunits.

N-terminal Portion of ORC3 Interacts with the C-terminal Portion of ORC2—Upon establishing the fact that ORC2 and ORC3 form a core complex, we mapped the interacting domains of ORC2 and ORC3. N-terminal fragments of ORC3 labeled with [^{35}S]methionine were produced by *in vitro* transcription and translation in rabbit reticulocyte lysate. The proteins were incubated with bacterially expressed and purified GSTORC2 protein. Three polypeptides derived from ORC3 were capable of binding GSTORC2, whereas the control GST protein did not bind any of them (Fig. 4*A*). The smallest fragment that bound to ORC2 contained 200 amino acids from the N terminus of ORC3 (*construct 3*, ORC3N). To map the portion of ORC2 involved in the interaction with ORC3, we expressed and purified GSTORC2C, containing the C-terminal 225 amino acids of ORC2. Both full-length ORC3 and ORC3N bound to GSTORC2C (Fig. 4*B*), whereas control GST alone did not bind to any one of them (data not shown). Therefore, the C-terminal 225 residues of ORC2 interact with the N-terminal 200 residues of ORC3 to form the ORC2-3 complex at the core of human ORC. In the reciprocal deletion, removal of the first 200 amino acids of ORC3 abolished its ability to bind to GSTORC2 (Fig. 4*C*). Based on these results we conclude that N-terminal 200 residues of ORC3 are necessary and sufficient to interact with ORC2.

N-terminal 200 Amino Acids of ORC3 Can Compete with the Full-length ORC3—If the N-terminal 200 residues of ORC3 are sufficient for binding ORC2, ORC3N might be able to compete with full-length ORC3 for binding to GSTORC2. [^{35}S]Methionine-labeled ORC3 was bound to GSTORC2 beads under conditions where the latter was limiting. These beads were then incubated with increasing amounts of ORC3N. We found that ORC3N protein could compete with the full-length ORC3 protein for association with GSTORC2 (Fig. 5).

ORC3N Cannot Form a Complex That Contains ORC4-5—Next we asked whether ORC3N is capable of mediating the interaction of ORC2 with ORC4 and ORC5. Sf9 insect cells were infected with baculoviruses expressing GSTORC5, ORC2, ORC4, and ORC3N. After affinity purification on glutathione beads and Western blotting, we confirmed the presence of GSTORC5 in the eluate from the beads. Interestingly, in contrast to the result in Fig. 1*A*, none of the other ORC subunits came down with GSTORC5, although they were all present in the input lane at reasonable quantities (Fig. 6*A*). We confirmed the physical interaction between ORC2 and ORC3N in the insect cell lysates by co-immunoprecipitation reactions. The lysate was immunoprecipitated using either anti-ORC2 or anti-

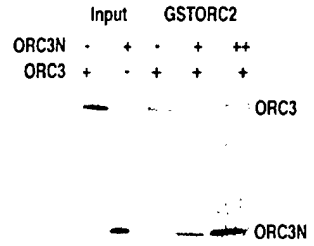

FIG. 5. **ORC3N can compete with full-length ORC3.** GSTORC2 beads were incubated with *in vitro* transcribed and translated full-length ORC3. After incubation, beads were thoroughly washed using binding buffer and incubated again with increasing amount of *in vitro* transcribed and translated ORC3N200. Beads were finally washed, and bound labeled proteins were visualized by SDS-polyacrylamide gel electrophoresis followed by fluorography.

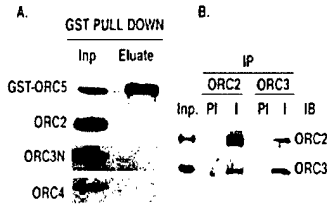

FIG. 6. **ORC3N does not form a complex with ORC2, -4, and -5.** *A*, Sf9 insect cells were infected with baculoviruses expressing GSTORC5, -2, -4 and ORC3N200. Proteins bound to glutathione-agarose beads were immunoblotted using either anti-GST or anti-ORC antibodies. *B*, immunoprecipitation using anti-ORC2 and anti-ORC3 antibodies. Insect cell lysate from *A* was immunoprecipitated (*IP*) using either anti-ORC2 or anti-ORC3 antibodies followed by immunoblotting (*IB*) with either anti-ORC3 or anti-ORC2 antibodies. In each case 5% of the lysate (used for immunoprecipitation) was loaded in the input (*Inp*) *lanes*. Sera used for immunoprecipitation: *I*, immune, and *PI*, preimmune.

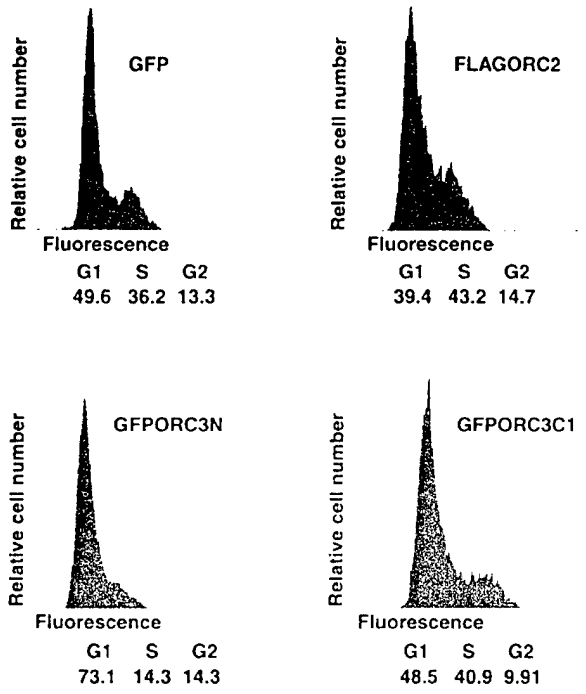

FIG. 7. **FACS analysis of U2OS cells transfected with different constructs.** U2OS cells were transfected either with farnesylated GFP (CLONTECH) or in combination with FLAGORC2, GFPORC3N, or GFPORC3C1. Transfected cells were fixed and stained with propidium iodide and then analyzed by FACS. The percentage of cell population present at different cell cycle stages in each transfection is shown at the bottom of the each *panel*.

ORC3 antibodies followed by immunoblotting with both the antibodies. ORC2 was detected in anti-ORC3 immunoprecipitate and vice versa (Fig. 6*B*). Therefore, ORC3N is capable of interacting with ORC2, but this interaction was not sufficient for further binding of ORC4 and ORC5. The C-terminal portion of ORC3 appears to be crucial for binding of ORC4 and ORC5 subunits to ORC2-3 subcomplex.

Expression of ORC3N in U2OS Cells Causes Cell Cycle Arrest—Because ORC3N did not form a complex with ORC4 and ORC5 but still could interact with ORC2, we reasoned that ORC3N might show a dominant negative effect on the cell cycle if overexpressed in a human cancer cell line. ORC3N and ORC3C1 were cloned into GFPC1 (CLONTECH) expression vector to produce non-farnesylated GFP fusion proteins. U2OS cells were transfected with plasmids expressing farnesylated GFP alone or in combination with FLAGORC2 or GFPORC3N or GFPORC3C1 (1:3 molar ratio) followed by FACS analysis after 48 h. Cells transfected with GFP alone, FLAG-ORC2, or GFP-ORC3C1 showed normal cell cycle progression, whereas cells transfected with GFP-ORC3N were blocked mostly in G_1 (73%) (Fig. 7). This is the first evidence for any cell cycle effect of any human ORC protein. Since ORC3N can still bind ORC2 but not ORC4 and ORC5, it is possible that over-expressed ORC3N interacts with ORC2 but prevents functional ORC formation. Consistent with this, over-expression of full-length ORC3, which interacts with ORC2 but allows functional ORC formation, did not block the cells in G_1 (data not shown).

DISCUSSION

We report here that human ORC2, -3, -4, and -5 form a core complex in baculovirus-infected insect cells. ORC1 and ORC6 did not interact with this core complex under these experimental conditions. This was confirmed by using different tags on

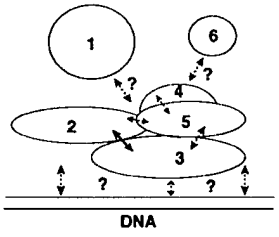

FIG. 8. **Model of human ORC2–5 subcomplex.** Human ORC subunits are showed as numbers 1–6. ORC2–5 subunits are shown to form a complex. N and C in ORC2 and -3 depict N- and C-terminal residues of the individual subunits. The *arrow* with the *bold line* shows a strong interaction, whereas *arrows* with *dotted lines* indicate weak interactions.

different ORC subunits and is consistent with our previously published data showing that endogenous ORC2, -3, -4, and -5 in a HeLa cell extract physically interacted with each other but not with ORC1 and -6(14). Recently ORC1 has been shown to interact with ORC2 by co-immunoprecipitation reaction using high salt nuclear lysate from HeLa cells (21). This study did not include ORC3-6 proteins. Unfortunately, we could not reproduce the co-immunoprecipitation of ORC1 with ORC2 in human cell lines using two different ORC1 antibodies, raised independently. Under the same conditions we still found strong interaction among ORC2–5 subunits. The data from HeLa cell extract and recombinant baculovirus proteins strongly suggest that ORC2–5 form a core complex with ORC1 and ORC6, joining the complex either at very restricted times or locations or in a very labile interaction that is easily disrupted upon cell lysis.

We further found that ORC2 and ORC3 form a tight complex essential for binding ORC4 and ORC5. Gel filtration of 293T cell extract showed that ORC2 and 3 were the only two subunits that

mostly co-eluted, consistent with the direct interaction between ORC2 and -3 reported here (16). Under our experimental conditions, none of the other ORC subunits interact with each other directly. The N-terminal 200 amino acids of ORC3 were enough to interact with C-terminal portion of ORC2 but not sufficient to allow association with ORC4 and ORC5, suggesting that the C-terminal of ORC3 is required for ORC4 and ORC5 loading on ORC2-3 subcomplex.

Recently, in yeast *S. cerevisiae*, ORC2 and ORC3 have been shown to interact directly (22). Insect cells were infected with baculoviruses expressing yeast ORCs, and recombinant yeast ORC was purified and tested for its DNA binding ability. ORC6 was found dispensable for DNA binding property. Elimination of ORC3 during baculovirus infection led to formation of ORC sub-complex without the presence of ORC2, suggesting that yeast ORC3 recruits ORC2 to the complex. Likewise, yeast ORC4 and ORC5 were shown to interact with each other. In addition, when yeast ORC was bound to yeast ARS1, ORC1, -2, -4, and -5 subunits were shown to directly contact ARS1 DNA by UV cross-linking (22). The human ORC2–5 sub-complex, however, did not show any sequence-specific DNA binding activity (data not shown).

Finally we showed that ORC3N has a dominant negative effect on cell cycle progression. U2OS cells expressing the same fragment were blocked in G_1, whereas the ORC3C1 or ORC2 protein did not prevent the cells from normal cell cycle progression. This can be explained by the fact that ORC3N titrates out ORC2 or an unknown cellular protein in the cell, thereby blocking G_1-S transition. Given that ORC2 and ORC3N form a very tight complex but the latter cannot support ORC2, -3, -4, -5 complex formation, we believe that ORC2 is the target that is titrated out by ORC3N. We cannot, however, overcome the effect of ORC3N by over-expressing ORC2 or ORC2C.[2] ORC2 or ORC2C might not be expressed at high enough levels to titrate out the ORC3N. Alternatively, the ORC3N targets an unknown cellular factor to cause the G_1-S block.

Based on these results we propose a molecular architecture of human ORC (Fig. 8). ORC2 and ORC3 interact directly with the C terminus of the former subunit, in close proximity with the N terminus of the latter. This binding favors the loading of ORC4 and -5 subunits via the C-terminal residues of ORC3. Although none of the other ORC subunits interacted with each other, we cannot rule out weak inter-subunit interactions among themselves. Further experiments will address how the ORC2–5 complex recruits ORC1 and ORC6 to form human ORC and study how the complex interacts with DNA.

[2] S. K. Dhar and A. Dutta, unpublished information.

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Replication from oriP of Epstein-Barr Virus Requires Human ORC and Is Inhibited by Geminin

Suman K. Dhar,[1,5] Kenichi Yoshida,[1,5]
Yuichi Machida,[1] Poonam Khaira,[2]
Biswendu Chaudhuri,[3] James A. Wohlschlegel,[1]
Michael Leffak,[2] John Yates,[3] and Anindya Dutta[1,4]
[1]Department of Pathology
Brigham and Women's Hospital and Harvard Medical School
Boston, Massachusetts 02115
[2]Department of Biochemistry and Molecular Biology
Wright State University
Dayton, Ohio 45435
[3]Department of Cancer Genetics
Roswell Park Cancer Institute
Buffalo, New York 14263

Summary

A hypomorphic mutation made in the *ORC2* gene of a human cancer cell line through homologous recombination decreased Orc2 protein levels by 90%. The G1 phase of the cell cycle was prolonged, but there was no effect on the utilization of either the c-Myc or β-globin cellular origins of replication. Cells carrying this mutation failed to support the replication of a plasmid bearing the oriP replicator of Epstein Barr virus (EBV), and this defect was rescued by reintroduction of Orc2. Orc2 specifically associates with oriP in cells, most likely through its interaction with EBNA1. Geminin, an inhibitor of the mammalian replication initiation complex, inhibits replication from oriP. Therefore, ORC and the human replication initiation apparatus is required for replication from a viral origin of replication.

Introduction

Origin-recognition Complex (ORC) was identified in *S. cerevisiae* as a complex of proteins essential for the initiation of DNA replication (Bell and Stillman, 1992). ORC has subsequently been identified in other species and shown to be functionally important for DNA replication during development in *Drosophila* (Austin et al., 1999; Chesnokov et al., 1999; Gossen et al., 1995; Landis et al., 1997) and in *Xenopus* egg extracts (Carpenter et al., 1996). Individual ORC subunits have been identified in humans and other mammals (Dhar and Dutta, 2000; Gavin et al., 1995; Pinto et al., 1999; Quintana et al., 1997; Thome et al., 2000; Tugal et al., 1998), but their role in mammalian cells is still unclear because of the absence of appropriate genetic or biochemical systems for such study.

Because ORC is expected to be essential for viability, a standard gene knockout approach in mouse is unlikely to give much information beyond the fact that the genes are essential for normal development. A potential solution to this problem is to develop either conditional or hypomorphic mutations in ORC genes in mammalian cells. We have reported that hypomorphic mutations in *Drosophila ORC3* produced enough Orc3 protein to allow viability but exhibited a specific proliferation defect in midbrain neuroblasts during development (Pinto et al., 1999). Another recent development has been the use of homologous recombination to alter chromosomal genes in mammalian somatic cells in culture (Chan et al., 1999; Lengauer et al., 1997; Waldman et al., 1995). In this paper, we combine these ideas to engineer a hypomorphic mutation in the human *ORC2* gene in a cancer cell line.

The function of ORC is best understood in yeast and *Xenopus* egg extracts and is connected to "replication licensing," the mechanism by which eukaryotic DNA replication is limited to once per cell cycle (Blow and Laskey, 1986; Dutta and Bell, 1997; Laskey and Madine, 1996; Stillman, 1996). ORC is bound to origins of DNA replication throughout the cell cycle. In G1 phase, ORC recruits CDC6 and Cdt1, which in turn recruit the MCM complex to form a prereplicative complex (pre-RC) (Cocker et al., 1996; Diffley et al., 1995). At the G1-S transition, the action of cyclin-dependent kinases promotes the loading of CDC45, RPA, and DNA polymerases at the replication origin and DNA synthesis is initiated. The increased activity of cyclin-dependent kinases prevents a second round of replication initiation at origins that have already initiated one round of DNA synthesis. In addition, a protein called geminin appears in the cell to inhibit Cdt1 to provide a second mechanism by which rereplication is prevented (McGarry and Kirschner, 1998; Tada et al., 2001; Wohlschlegel et al., 2000). Only after cells have passed through mitosis and entered the next G1 are the cyclins and geminin degraded to allow the reassembly of pre-RCs for the next round of DNA synthesis.

Epstein-Barr virus (EBV) is a 165 kb double-stranded DNA virus of the herpesvirus family that replicates as an episome in latently infected cells. B cells or epithelial cells are latently infected by EBV in 90% of humans and can cause carcinomas and lymphoproliferative disease in immunosuppressed patients. To prevent this problem, it is essential that we learn how these viral episomes replicate in human cells and devise strategies to interfere with the process. A 1.7 kb region of the EBV chromosome, oriP, supports the replication and maintenance of recombinant plasmids in human cells in the presence of a single EBV-encoded protein, EBNA-1 (Yates et al., 1985). Replication initiates at or near a 120 bp component of oriP called DS (dyad symmetry), which binds EBNA-1 and is the functional replicator of oriP (Gahn and Schildkraut, 1989; Harrison et al., 1994; Yates et al., 2000). Since EBNA-1 appears to lack enzymatic activity (Frappier and O'Donnell, 1991; Middleton and Sugden, 1992), initiation of DNA replication at oriP must rely on cellular proteins. Plasmids bearing oriP are replicated no more than once per cell cycle (Yates and Guan, 1991), suggesting that the plasmids are controlled by replication licensing.

[4]Correspondence: adutta@rics.bwh.harvard.edu
[5]These authors contributed equally to this work.

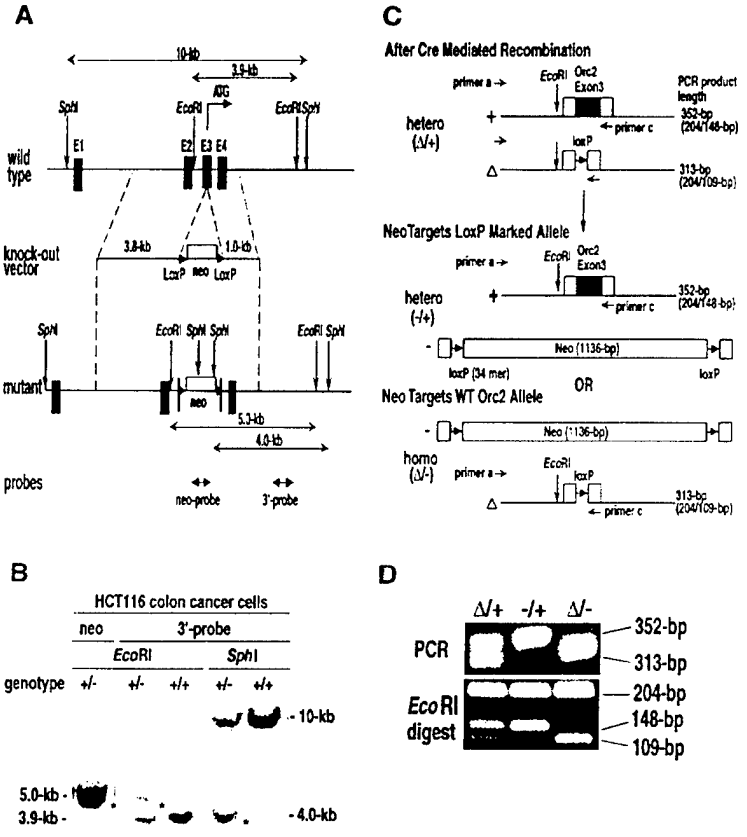

Figure 1. Targeted Disruption of the Human ORC2 gene

(A) WT ORC2 locus with the targeting vector and the resulting mutated locus. Solid boxes represent the first 4 exons. Probes used for Southern blot analysis are indicated. Recombination between the vector and endogenous locus deletes 72 bp of exon 3 containing the ATG.

(B) Southern blot of EcoRI- or SphI-digested genomic DNA from targeted and wild-type HCT116 cells hybridized with the probes shown in (A). The asterisks indicate DNA fragments specific to the mutated (−) allele: a 5 kb EcoRI fragment recognized by the neo probe or the 3′ probe and a 4 kb SphI fragment recognized by the 3′ probe.

(C) Cre-mediated recombination of the −/+ cells obtained in (B) produces a Δ/+ cell where the excision of the Neo cassette leaves a loxP site in exon 3 (Δ). The second round of targeting can either create a −/+ cell or a Δ/− cell. PCR with indicated primers "a" and "c" produce the diagnostic PCR products shown to the right of each allele. EcoRI digestion of the PCR products yields the fragments in parentheses.

(D) Genomic PCR of the cell clones to identify products of the second round of homologous recombination. Genomic DNA prepared from parental Δ/+ clone and from −/+ or Δ/− product clones were subjected to PCR with primer pairs "a" and "c" shown in (C). Gel electrophoresis and ethidium bromide stain to visualize the products of the PCR reaction (top) or the fragments produced by EcoRI digestion of the PCR products (bottom).

In this paper, we show that the cells with a hypomorphic mutation in human *ORC2* survive with only a slight prolongation of the G1 phase of the cell cycle. They do not permit EBNA1-dependent replication of episomes from oriP, but this replication is restored upon expression of wild-type Orc2 in these cells. Orc2 in cells is specifically associated with oriP and coimmunoprecipitates with EBNA1. Finally, cotransfection with geminin suppresses replication from oriP and this is rescued by overexpression of Cdt1. Taken together, this data suggests that ORC and other human initiation factors are required for replication from oriP. This is demonstration of the use of eukaryotic replication proteins in the initiation of DNA replication from a viral origin of replication. The result concurrently identifies a novel means by which to inhibit latent infection of Epstein Barr virus for therapeutic purposes.

Results

Mutation of ORC2

Homologous recombination was used to replace the third exon of *ORC2* (encoding the initiator ATG) in HCT116 colon carcinoma cells with a Neomycin phosphotransferase (NEO) gene (Figure 1A). *ORC2* +/− clones were identified in 2%–3% of G418-resistant colonies by Southern blotting (Figure 1B). Cre-mediated recombination of the *loxP* sites flanking the NEO gene excised the NEO cassette, made the cells susceptible to G418, and left a loxP site in place of most of exon3 in the allele called Δ-*ORC2*. (Figure 1C; Δ/+).

The second allele of *ORC2* was targeted with the same vector followed by G418 selection. Retargeting of the *loxP* marked allele (Δ) recreates the − allele in cells called −/+ (Figure 1C). PCR screening of genomic DNA with the indicated primers produces a 352 bp product from the + allele that is cut by EcoRI into fragments of 204 and 148 bp. Targeting of the wild-type *ORC2* allele, on the other hand, creates the desired cell-line (Δ/−) (Figure 1C). PCR screening produces a 313 bp product from the Δ allele that is cut by EcoRI into fragments of 204 and 109 bp. PCR analysis identified 8.8% of the G418-resistant clones as Δ/− mutants at the *ORC2* locus (Figure 1D, Δ/−).

The Δ-ORC2 Allele Produces a Low Level of an N Terminally Truncated Orc2 Protein

In a conventional gene deletion experiment, transcription and polyadenylation of the drug resistance cassettes inserted in the two alleles of the target gene prevents residual expression of the latter. In the strategy employed here, however, only a *loxP* site is left in the middle of exon 3 in the Δ allele. Although the initiator ATG is removed, low levels of a variant ORC2 mRNA

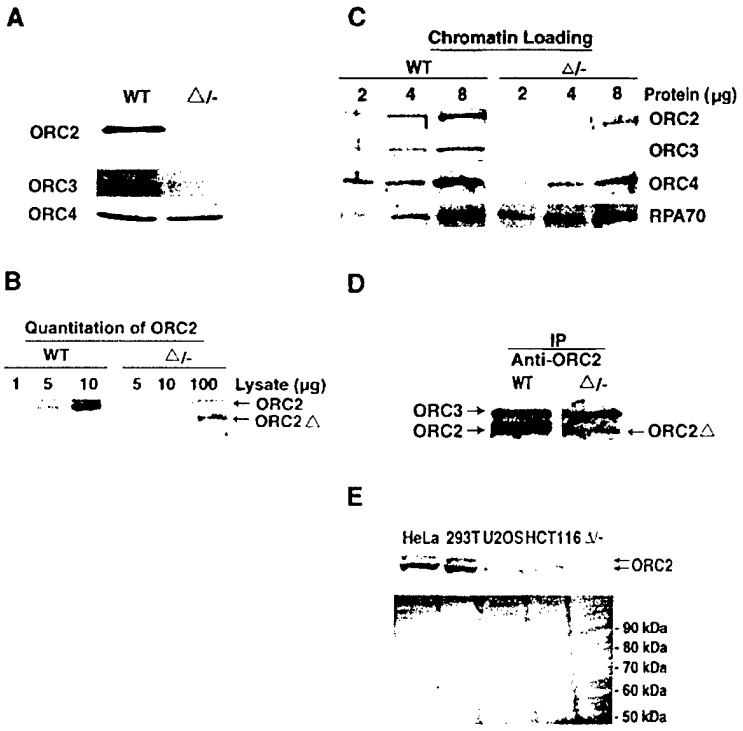

Figure 2. Biochemical Characterization of ORC in the Δ/− Cells

(A) Western blot analysis of total cell lysates using α-Orc2, α-Orc3, and α-Orc4 antibodies. Five micrograms of lysates of WT (+/+) or Δ/− cells loaded per lane.

(B) Indicated amounts of cell lysates were loaded to quantitate the low level of the 68 kDa ΔOrc2 protein in the lysates of Δ/− cells.

(C) Detection of various replication proteins in the chromatin fraction of WT and Δ/− cells. Two, four, and eight micrograms of protein from the chromatin fraction immunoblotted with antibodies to indicated proteins.

(D) Immunoprecipitation of 100 μg of chromatin proteins from WT and Δ/− cells with α-Orc2 antibody followed by immunoblotting with antibodies to Orc2 and Orc3.

(E) Immunoblotting of 10 μg of cell lysates from 293 (human embryonic kidney), HeLa (cervical carcinoma), U2OS (osteosarcoma), and HCT116 (colon carcinoma) cell lines with α-Orc2 antibody. Ponceau S stain is shown as a loading control.

produced by read-through transcription or alternative splicing could express an N terminally deleted Orc2 protein from methionine-74 encoded by exon 4. Although an α-Orc2 antibody did not recognize the full-length 72 kDa Orc2 protein in the Δ/− cells (Figure 2A), an Orc2-related polypeptide of 68 kDa was detected when 10 times more cell lysate was analyzed (Figure 2B). Because the 68 kDa protein (ΔOrc2) was seen only in cells with the Δ*ORC2* allele, the polypeptide was most likely the product of the *loxP*-marked Δ-*ORC2* locus.

Effect of the Hypomorphic Mutation in ORC2 on the Other ORC Subunits

The Δ/− cells express ΔOrc2 protein at 10% of the level of full-length Orc2 seen in wild-type (WT) cells. The level of Orc4 was unchanged in the Δ/− cells, while Orc3 levels were markedly decreased (Figure 2A). ORC3 mRNA is expressed at normal levels in the Δ/− cells (data not shown), suggesting that the decrease of Orc3 protein is due to posttranscriptional mechanisms. Previous studies have shown that Orc3 and Orc2 are tightly bound to each other in cells (Thome et al., 2000). Therefore, it is possible that the decrease in ΔOrc2 protein in the Δ/− cells results in free Orc3 that is unstable and subsequently degraded.

Immunoblotting different concentrations of protein from the chromatin fraction of cells indicated that the amounts of chromatin-associated ΔOrc2, Orc3, and Orc4 were decreased to less than 25% in the Δ/− cells (Figure 2C and data not shown). The DNA replication factor RPA70 is present on the chromatin at normal levels in the Δ/− cells, providing a positive control for chromatin recovery. We also show that the 68 kDa protein in Δ/− cells was immunoprecipitated by anti-Orc2 antibody and coimmunoprecipitated with Orc3, supporting the hypothesis that it is derived from the Δ-*ORC2* locus (Figure 2D).

The Hypomorphic Mutation in ORC2 Prolongs G1 Phase of the Cell Cycle

The proliferation rate of the *ORC2* Δ/− cells was decreased by 30% (doubling time 43 hr versus 33.5 hr in *ORC2* +/+ or +/− cells) (Figure 3A). There was no increase in apoptosis or senescence in the Δ/− cells (data not shown). FACS to measure the DNA content of asynchronously growing cells indicated a moderate increase in the G1 phase population in the Δ/− cells (Table 1), suggesting a prolongation of G1. Consistent with this finding, the percentage of cells entering S phase in Δ/− cells in the 24 hr following release from a mimosine induced G1-S block (Gilbert et al., 1995) is about half that in the WT cells (Figure 3B). [^{3}H]thymidine incorporation over 3 days in two different clones of Δ/− cells was also 30%–40% that of WT cells (Figure 3C). [^{3}H]thymidine incorporation in cultures released from a mimosine induced G1-S block showed that the height of the incorporation curve is decreased to 60%–70% without any change in the width of the curve (Figure 3D). This suggests that fewer cells enter S phase when Orc2 activity is compromised, but DNA replication is normal once the cells enter S phase so that the duration of S phase is unchanged. Based on these four different assays for cellular proliferation, we find that Δ/− cells proliferate at approximately 50% of the rate of WT cells. The slight differences between the results of these assays likely reflect the fact that different parameters are being measured. For example, the MTT assay in Figure 3A measures the metabolic activity of cells, while

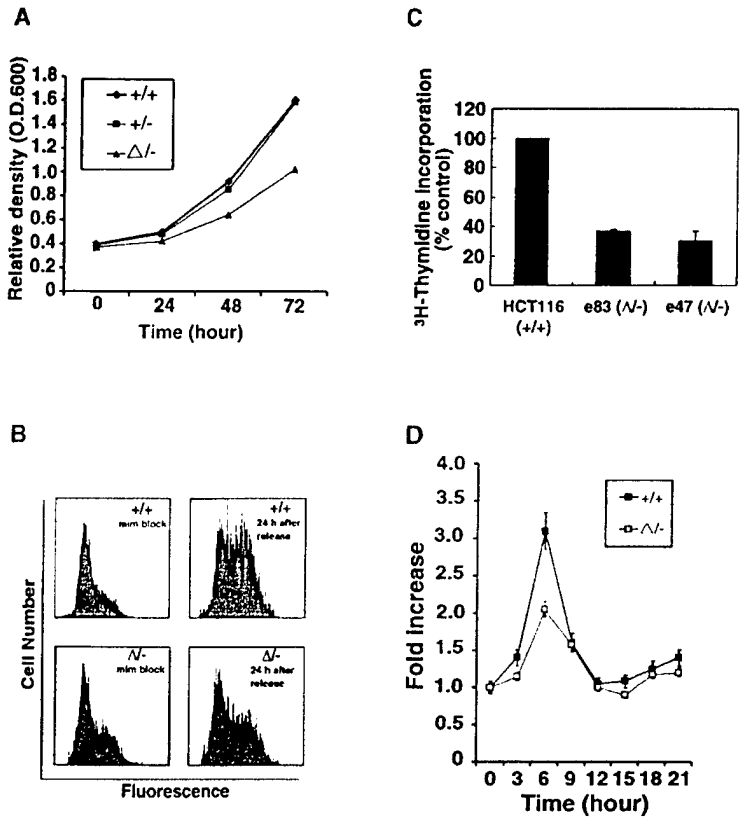

Figure 3. ORC2 Δ/− Cells Are Compromised in Cell Proliferation

(A) Cell proliferation of the indicated cell lines measured by MTT assay. Doubling times were determined by fitting the data to an exponential curve.

(B) FACS analysis for DNA content of +/+ (WT) and Δ/− HCT116 cells. Cells synchronized at the G1-S boundary by mimosine (left) and 24 hr following release from the mimosine block (right).

(C) [³H]thymidine incorporation of +/+ and two separate clones of Δ/− cells. Comparison of thymidine incorporation of asynchronous cells growing over 3 days in culture, with the incorporation in +/+ cells held at 100%. Mean and standard deviation of 4 measurements.

(D) Incorporation of thymidine during 1 hr pulses at indicated time points following release from mimosine block. The thymidine incorporation at each time point is normalized to the incorporation of that cell line in the 0 hr time point.

in Figure 3C, [³H]thymidine incorporation specifically measures DNA replication.

Replication Initiation Activity Is Unchanged at Two Chromosomal Origins of Replication

The lack of a significant prolongation of S phase suggests that in the Δ/− cells that do enter S phase, either the normal complement of origins is active, or the number of origins firing per chromosome can be significantly decreased without affecting the duration of S phase. To examine the initiation activity at chromosomal sites, the abundance of short nascent DNA strands was quantitated by PCR at two loci containing known origins of DNA replication, c-*myc* and β-globin (Figure 4). In both of these regions, the abundance and size distribution of DNA nascent strands suggests that there are multiple, nonrandom sites of replication initiation (Kamath and Leffak, 2001; Malott and Leffak, 1999; Tao et al., 2000). The pattern of nascent strand abundance at these specific initiation sites was unchanged between +/+ and Δ/− cells, suggesting that at least for these two origins, replication initiated normally in the Δ/− cells that do enter S phase.

Table 1. Percentage of Cells in Different Phases of the Cell Cycle (Mean and Standard Deviation of 3 Determinations for WT Cells and 6 Determinations for Δ/−cells)

HCT116 cells	G1	S	G2/M
WT	24.4 (1.2)	51.7 (1.9)	23.9 (1.1)
Δ/−	33.8 (3.2)	42.2 (1.6)	24.0 (2.3)

Abundance of Orc2 in Several Cell Lines

To ensure that the HCT116 cells did not contain atypically high levels of Orc2, we compared levels of Orc2 in four commonly used cancer cell lines (Figure 2E). Orc2 levels in HCT116 colon cancer cells were about half that in 293 or HeLa cells. Therefore, the excess of Orc2 relative to what is essential for chromosomal replication is a feature of several cancer cells.

The Hypomorphic Mutation in ORC2 Impairs Replication from the oriP of EBV

To test whether cellular Orc2 is required to support EBV replication, a plasmid carrying oriP, EBNA-1 and a hygromycin resistance gene (p220.2) was transfected into WT and Δ/− cells (Figure 5A). Replication of p220.2 allowed hygromycin resistant colonies to emerge in the WT cells but not in the Δ/− cells. DpnI-resistant plasmids were recovered from these colonies indicating that the transfected plasmids had replicated in the WT cells (Figure 5B, lane 1). pBabe-Puro, a plasmid that does not have oriP or EBNA-1 and is integrated in the host cell chromosome was used as a control and produced the same size and number of puromycin-resistant colonies in WT and Δ/− cells (Figure 5A). Therefore, the +/+ and Δ/− cells are not inherently different in their transfection efficiency or in their expression of drug resistance markers. The observation that the size and number of the puromycin-resistant colonies is similar in WT and Δ/− cells also indicates that the inability of the plasmid p220.2 to replicate cannot be due to the difference in proliferation rates between the two cell lines.

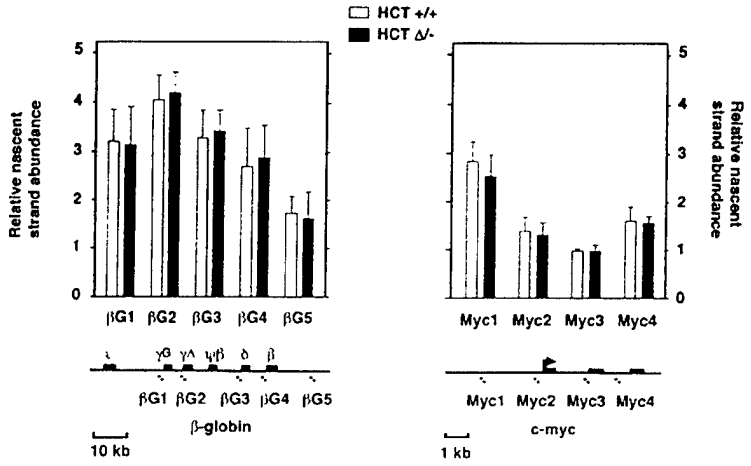

Figure 4. Relative Abundance of Nascent DNA Strands in the c-Myc and β-Globin Loci: Comparison of WT (+/+) HCT116 Cells and Δ/− HCT116 Cells

The abundance of 1–2 kb nascent DNAs at four sequence-tagged sites in the c-Myc locus and at five sequence-tagged sites in the β-globin locus in wild-type (+/+) and ORC2 mutant (Δ/−) cells. Maps show the c-Myc P_1 promoter (arrow) and exons (boxes), and the cluster of globin genes (boxes) and locations of the primers used. Means and standard deviations of 6–12 PCRs on each of three or four independent nascent DNA preparations from each cell type. Values are normalized to the abundance at Myc3.

To confirm that the Δ/− cells did not support replication from oriP, a transient replication assay was performed (Figure 5B). p367 (with wild-type oriP and expressing EBNA-1) (Yates et al., 2000) was transfected into WT and Δ/− cells. Ninety-six hours after transfection, DpnI-resistant (replicated) p367 plasmids were detected by Southern blotting (Figure 5B, lanes 6 and 7). Quantitation of the DpnI-resistant DNA in the blots (Figure 5C) showed that replication was decreased to less than 10% in the Δ/− cells compared to WT cells, suggesting that ORC was necessary for efficient replication from oriP. As in the stable replication assay, the fact that the replication of the oriP-containing plasmids was reduced by 90% while the proliferation rate between Δ/− and WT cells differs only by 50% also strongly suggests that the inability of the oriP plasmid to replicate cannot be attributed to the slower growth rate of the Δ/− cells.

If the defect in OriP-based plasmid replication in Δ/− cells is due to the mutation in ORC2, expression of wild-type Orc2 protein should rescue the replication defect. Infection of HCT116 Δ/− cells with an adenovirus vector that expresses Orc2 resulted in the reappearance of wild-type 72 kDa Orc2 protein in the cell extracts (Figure 5D). Following infection with Adeno-Orc2 or with negative control Adeno-GFP (expressing green fluorescent protein), the HCT116 Δ/− cells were transfected with p367 (Figure 5E). The increase in DpnI-resistant plasmid DNA in lane 4 (compared to lane 3) and the quantitation of the result (Figure 5F) indicates that the transient expression of wild-type Orc2 in Δ/− cells partially restored replication from OriP.

The failure of Δ/− cells to support replication of an OriP-based plasmid could be because of the low level of Orc2 or because ΔOrc2 is missing a critical region essential for EBV replication. Cotransfection of p367 with a plasmid overexpressing FLAG-tagged ΔOrc2 rescued replication from OriP effectively (data not shown), suggesting that the defect in Δ/− cells was due to the low level of Orc2 and not a qualitative defect in the ΔOrc2 protein.

Orc2 Is Specifically Associated with the oriP of EBV Chromosomes in Cells

The above results suggest that ORC has a positive role in replication from oriP. To investigate whether ORC associates physically with oriP, chromatin immunoprecipitation (ChIP) assays were performed on chromatin prepared from human 293 cells that carried multiple copies of a 12.6 kb oriP-dependent plasmid (p818, Figure 6A). DNA was recovered from chromatin precipitated by antibodies specific to EBNA-1 or Orc2, or by nonimmune serum, and analyzed by PCR for four regions of the plasmid simultaneously. The four regions of the plasmid were amplified by PCR using DNA from de-cross-linked chromatin (lanes 7–9). The results indicate that chromatin containing the DS region of the plasmid was specifically precipitated by antibodies against Orc2 (lanes 1–2), as well as EBNA-1 (lanes 5–6), but not by nonimmune serum (lanes 3–4). The nonimmune sera used for this experiment included antibodies against a splicing factor, Upf1, and against an EBV-encoded protein, LMP-2. The data indicate that Orc2, and by extension ORC, is physically linked to DS, the replicator of oriP.

Orc2 Interacts with EBNA1

If ORC and proteins downstream from ORC are required for replication from oriP, we hypothesized that the ORC is recruited to the latter through the viral protein EBNA1. Immunoprecipitation of lysates of DG75 cells carrying p818 with anti-Orc2 antibody coimmunoprecipitated a small but reproducible amount of EBNA1 (Figure 6B). Therefore, ORC is most likely recruited to oriP via protein-protein interactions with EBNA1.

Geminin Inhibits Replication from oriP of EBV

If ORC is required for replication from oriP, we reasoned that other components of the replication initiation complex might also be required. Geminin, a regulator of the cell cycle, interacts with one of these downstream components, Cdt1, to prevent the loading of MCM proteins (Wohlschlegel et al., 2000). Geminin is degraded by the anaphase-promoting complex and can be stabilized by mutation of a specific destruction box (geminΔDB) (McGarry and Kirschner, 1998). p367 (a plasmid containing oriP and EBNA1) was cotransfected into HCT116 cells with plasmid pEBG expressing glutathione S transferase (GST) as a control or with plasmids expressing GST-geminin or GST-geminΔDB (Figure 7A). Judging from the amount of DpnI resistant replicated

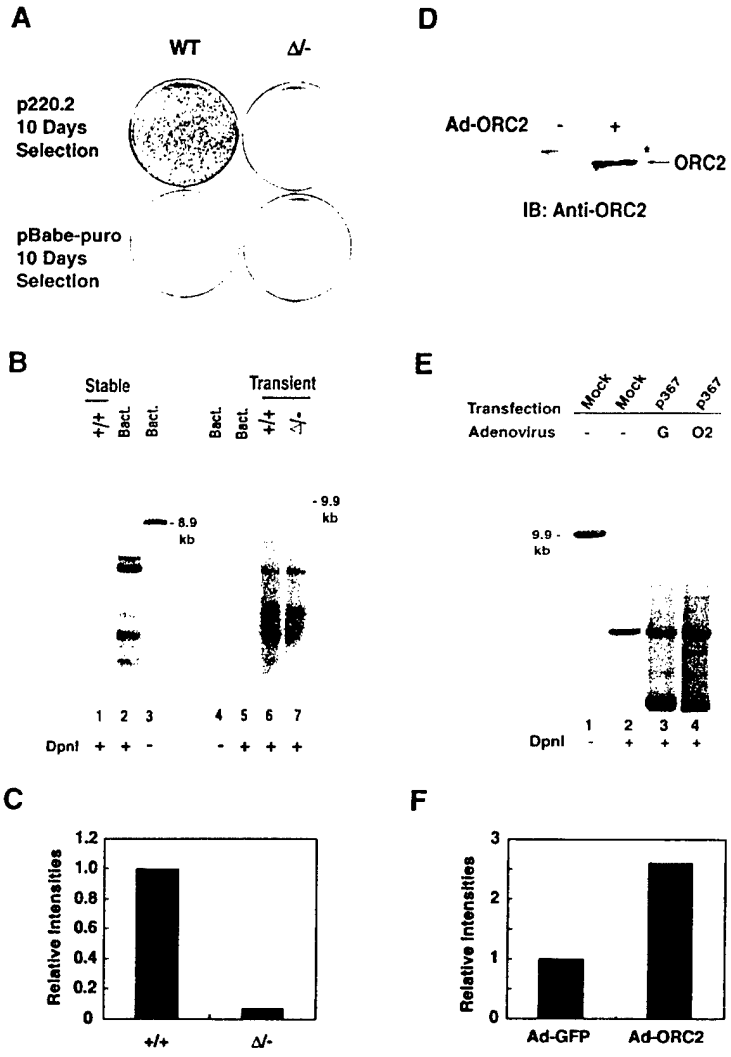

Figure 5. Orc2 Δ/− Cells Are Defective in DNA Replication from oriP of EBV and This Defect Is Rescued by Expression of Wild-Type Orc2

(A) Establishment of drug-resistant colonies of WT and Δ/− cells after transfection of plasmid p220.2 carrying EBV oriP, EBNA-1 and the hygromycin resistance marker or of pBabe-Puro, a plasmid containing a puromycin resistance gene as a control. Following selection in hygromycin (top) or puromycin (bottom), colonies were visualized by staining with crystal violet.

(B) Southern Blot to detect DpnI-resistant plasmids that have replicated in mammalian cells. In indicated lanes, DpnI was used to digest unreplicated DNA retaining the *dam* methylation acquired in *E. coli*. Plasmid DNA in all lanes was linearized with BamHI and detected by probing with the entire plasmid. The plasmids were either obtained after transfection of HCT116 cells (+/+ or Δ/−) or from *E. coli* (Bact). Lane 1: Stable replication of p220.2 in hygromycin resistant colonies derived from WT HCT116 cells 10 days after transfection. Lanes 6–7: Transient replication of p367 96 hr following transfection of indicated cell lines. Lanes 2–5: Plasmid obtained directly from bacteria, mixed with cellular genomic DNA from mock-transfected cells and digested with indicated enzymes to provide size markers for DpnI-resistant plasmid (lanes 3 and 4) and control for complete DpnI digestion (lanes 2 and 5).

(C) Quantitation of replication in Δ/− cells (from Figure 5B). The relative intensities compare the normalized intensities of the DpnI resistant DNA in each cell line. The normalized intensity from HCT116 +/+ cells is held at 1.

(D) Δ/− HCT116 cells (left) and same cells 48 hr following infection with adenovirus expressing Orc2. Ten micrograms total cell lysate immunoblotted with α-Orc2 antibody. "*" indicates a background band indicating equal loading in the two lanes.

(E) p367 replication in Δ/− HCT116 infected with adenovirus expressing GFP (G, lane 3) or Orc2 (O2, lane 4). Lanes 1 and 2 contain bacterial p367 mixed with genomic DNA from mock-transfected Δ/− HCT116 cells. DNA in all lanes was linearized with BamHI, and in indicated lanes digested with DpnI.

(F) Rescue of oriP-based plasmid replication in Δ/− HCT116 cells by adenovirus expressing full-length Orc2 (from Figure 5E). The normalized intensity of replicated DNA in Δ/− cells is held at 1.

plasmid DNA, GST-geminin inhibits replication of p367 (lane 4). GST-gemininΔDB, which expresses a higher concentration of the protein (data not shown), inhibits replication from p367 to a greater extent (lane 5). Quantitation of the results confirms that geminin suppresses replication from OriP (Figure 7B).

We attempted to reverse the repressive effect of geminin by coexpressing Cdt1 (Figure 7C). Because an excess of Cdt1 (relative to geminin) is required for the rescue, and because of limits on the maximum amount of plasmid that can be transfected, the ratio of the geminin expressing plasmid to p367 (0.83) was lower than in Figures 7A and 7B (2.0) accounting for the weaker inhibition of replication by geminin. Cdt1 rescued inhibition by geminin (Figure 7C) but did not stimulate oriP replication when expressed alone (data not shown). Therefore, Cdt1 and other initiation factors downstream from ORC are likely to play a role in DNA replication from oriP.

Discussion

Null mutations of essential genes are difficult to study in mammalian cells, preventing the genetic evaluation of the DNA replication machinery of cancer cells. Here, we solve this problem by creating a hypomorphic mutation in the human *ORC2* gene in a cancer cell line. The results indicate that Orc2 plays a role in moving cells from G1 into S, but once in S, the Δ/− cells replicate chromosomal DNA normally. One possibility is that the assembly of pre-RCs is stochastic and the number of assembled pre-RCs must exceed a certain threshold before mammalian cells can enter S phase. At low concentration of ORC (in Δ/− cells), G1 phase is prolonged,

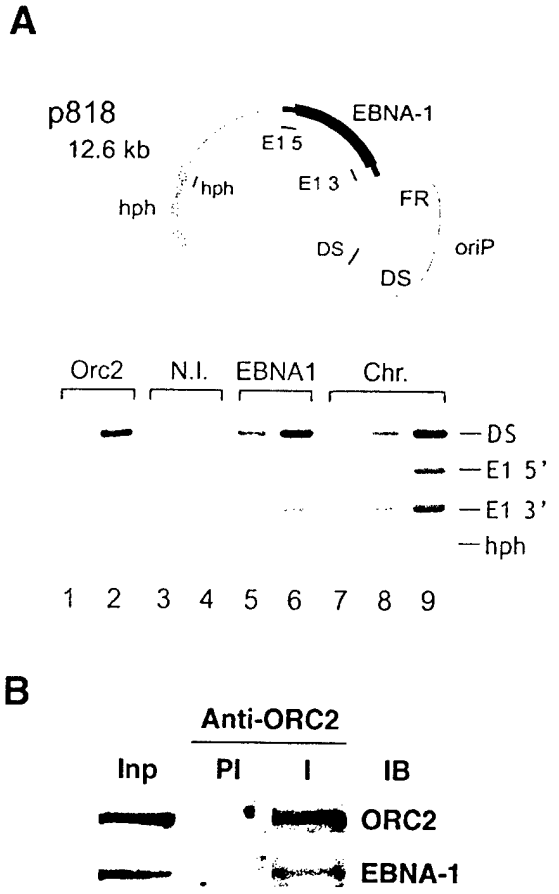

Figure 6. Association of ORC with oriP and EBNA-1

(A) Association of ORC with oriP, detected by ChIP. Cross-linked chromatin (30 μg) from 293 cells carrying the plasmid p818 (diagram, above) was precipitated using antibodies specific to EBNA-1, Orc2, or with nonimmune serum (N.I.), as indicated (gel image, below). Either 1/250 of the recovered DNA (lanes 2, 4, and 6) or 1/1250 (lanes 1, 3, and 5) was tested by PCR for four regions of the plasmid simultaneously. For lanes 7–9, 0.3 ng, 1.5 ng, and 7.6 ng, respectively, of the input chromatin were tested in parallel. Shown is the reverse image of an agarose gel stained with SYBR Gold. The amplified regions of the plasmid are indicated inside the circle of the diagram, and the PCR products, ranging from 182 bp to 435 bp, are indicated at the right of the gel image. Also indicated for p818 are the EBNA-1 gene and flanking sequences (black), oriP, its DS and FR components, and flanking sequences (light gray), and the hygromycin B-resistance gene (hph, dark gray).

(B) Immunoprecipitation of 250 μg of lysate from DG75 cells carrying p818 with α-Orc2 antibody and preimmune serum. The immunoprecipitates were immunoblotted with α-Orc2 antibody and with α-EBNA1 antibody OT1x. Input lanes contain 12.5 μg (for Orc2) and 25 μg (for EBNA1) of the cell lysate.

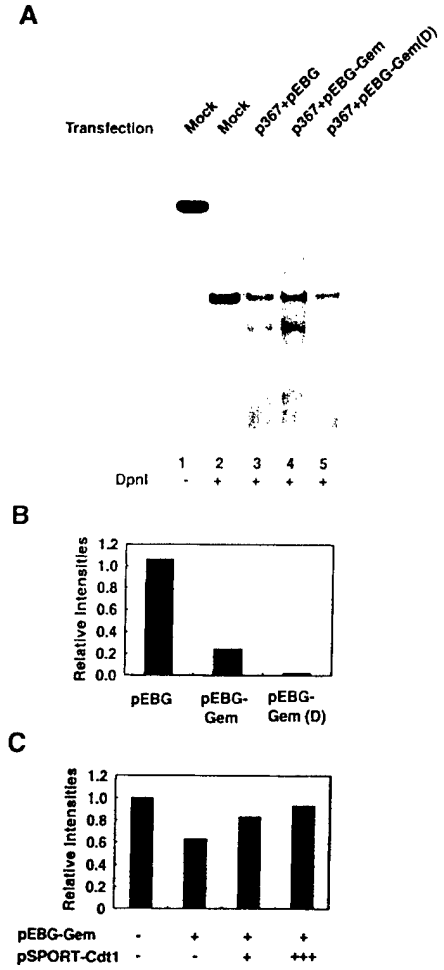

Figure 7. Geminin Inhibits Latent EBV Replication

(A) Geminin inhibits replication of EBV-based episome. Southern blot to detect DpnI-resistant p367 plasmids that have replicated in HCT116 +/+ cells following cotransfection of 2 μg of p367 with 4 μg each of plasmids expressing GST (lane 3), GST-geminin (lane 4), and GST-geminin-ΔDB (lane 5). Lanes 1 and 2 are the same as in Figure 5E.

(B) Inhibition of oriP-based replication by geminin (from Figure 7A). The normalized intensity of replicated DNA in cells transfected with EBG is held at 1.

(C) Rescue of geminin-induced inhibition by Cdt1. One microgram of EBG geminin and 1 or 3 μg of CMV-Cdt1 was cotransfected with 1.2 μg p367. Variable amounts of pcDNA3 were added to keep the total amount of DNA constant for each transfection.

as cells need more time to form enough pre-RCs to enter S. Once enough pre-RCs have been assembled to allow a successful S phase, DNA replication proceeds as in wild-type cells. Alternatively, ORC proteins have been postulated to play a role in chromosome structure and gene silencing (Ehrenhofer-Murray et al., 1995; Foss et al., 1993; Pak et al., 1997). The decrease in ORC in Δ/− cells might increase the expression of inhibitors of the G1-S transition, resulting in a prolongation of the G1 phase.

Although the Δ/− cells suffered a 90% decrease in the abundance of Orc2 protein and at least a 75% decrease in the amount of chromatin-bound ORC, there was only a minor effect on cellular replication as assessed by the duration of S phase and the origin activity of known cellular origins. This result suggests that the amount of Orc2 normally present in cells is in excess over what is required for DNA replication. In *S. cerevisiae*, removal of 90% of the known origins of replication from a chromosome still permits replication of the chromosome in the normal duration of S phase (Dershowitz and Newlon, 1993). We show that mammalian chromo-

somes could replicate normally with less than 25% of the normal complement of chromatin-bound replication initiator proteins. It should be noted, however, that although we failed to find any deficit in origin firing, this may be due to the specific origins chosen for analysis. We cannot completely rule out the possibility that fewer origins fire in the Δ/− cells compared to the WT cells.

In contrast to the minimal effects on chromosomal DNA replication, we provide clear evidence that cellular Orc2 is necessary for replication from oriP of EBV. The extreme sensitivity of the oriP-dependent plasmids to the *ORC2* mutation could be because an episome with one origin of replication is more sensitive to partial inhibition of replication initiation than a larger chromosome with multiple origins. EBNA1 and oriP are still essential for the replication of the EBV-based episomes in HCT116 cells (data not shown). The specific association of Orc2 with oriP and with EBNA1 supports the notion that ORC binds to the EBV plasmid near its origin of replication, probably through the EBNA1 protein, and plays a positive role in replication initiation activity. Additional work shows that Orc3 and Orc4 are associated with oriP in chromatin immunoprecipitation experiments (B.C. and J.Y., unpublished results). Orc2 could be recruited to additional sites where EBNA1 binds (like the FR in the oriP), but we have no evidence of this yet in the ChIP experiments. This might suggest that the stable association of ORC with DNA depends not only on the interaction with EBNA1 but also on additional interactions with adjoining DNA or proteins at specific sites on the oriP.

If a sequence-specific DNA binding factor like EBNA1 helps recruit ORC to oriP, recruitment of ORC to cellular chromosomal origins of replication might also be dependent on sequence-specific DNA binding factors. Such indirect recruitment of mammalian ORC to chromatin by diverse sequence specific factors might explain why it has been so difficult to find a single DNA sequence that acts as a replicator in mammalian chromosomal origins of replication.

Replication licensing is a mechanism by which cells ensure that chromosomal origins fire once and only once per cell cycle. EBV chromosomes in latently infected cells also replicate once and only once per cell cycle, but the mechanism for this regulation was unclear. The replication licensing mechanism operates at the level of pre-RC formation such that the assembly of pre-RCs is permitted in G1 phase but actively inhibited during the S, G2, and M phases of the cell cycle. The implication of ORC and other components of the pre-RC (Cdt1 by extension from geminin) in replication from oriP can now explain how viral chromosomes are subjected to the same replication licensing mechanism that controls cellular chromosomes.

The inhibition of replication from oriP by coexpression of geminin confirms by an independent mechanism that the EBV-based plasmid utilizes cellular replication initiation factors for DNA replication. Rescue of this inhibition by Cdt1 implicates this protein in a replication function in mammalian cells. Consistent with the fact that geminin inhibits the loading of MCM proteins on *Xenopus* chromatin, MCM proteins have also been detected on OriP in chromatin immunoprecipitation experiments (B.C. and J.Y., unpublished results). These results also suggest that geminin and agents that mimic the action of geminin could cure human cells of latent EBV infection. We believe that geminin-based therapeutics would have considerable advantages over other antiviral agents that inhibit DNA replication. Geminin would be unique among antiviral agents in its ability to inhibit the initiation of DNA replication but not elongation. Since episomes contain only one origin of replication while chromosomes contain multiple origins (not all of which are necessary for cell survival), it is likely that viral episomes would be far more sensitive to geminin-based drugs that specifically target initiation. This idea is supported by the fact that overexpression of geminin has only a minor effect on cell cycle progression of mammalian cells (data not shown), whereas viral replication is strongly inhibited. Antiviral agents which target the elongation machinery would not have this large therapeutic index, since elongation proteins are likely to be equally essential for cellular and viral replication.

The discovery that cellular replication initiator proteins are necessary to support EBV replication from oriP could also be an important step toward the treatment and prevention of EBV-associated neoplasias. Malignant cells from nasopharyngeal carcinomas, EBV-positive Burkitt's lymphoma, and Hodgkin's disease all carry one or more EBV episomes replicating without integration into the host genome. Since the viral genes that cause the malignancy are encoded by the EBV episome, elimination of the episome by treatment with geminin-based agents may help control the malignancy. Another interesting possibility is the use of geminin-based agents as prophylactics. Geminin-based preventive therapy can be used to eliminate latently infected cells in patients before they become immunosuppressed (due to progression of AIDS, posttransplant therapy, or therapy of autoimmune disease), to prevent the subsequent development of malignancy when immunosuppression does set in. Future experiments will explore these possibilities as well as examine whether ORC is required for lytic infections of EBV and other herpesviruses. EBV lacks a virally encoded replicative helicase. Therefore, as with replication from oriP, lytic replication from oriLyt might be dependent on the MCM helicase and be susceptible to geminin. In addition, we will test whether ORC and other cellular replication initiation factors are required for the chronic maintenance in human cells of other double-stranded DNA viruses.

Experimental Procedures

Orc2 Targeting Construct and Screening for Recombinants

A promoterless strategy was adapted for targeting the Orc2 gene (Waldman et al., 1995). A BAC clone (GenBank accession number AC005037) containing human Orc2 was obtained from Genome Sequencing Center at Washington University (St. Louis) and used as the source for homologous arms. The two PCR amplified fragments, one 3.8 kb and the second 1.0 kb, were used to construct the 5′ and 3′ arms of the targeting vector, respectively. The 3.8 kb subclone contained the region immediately 5′ of the initiation codon located in exon 3 of the Orc2 coding region. The 1.0 kb subclone contained a region beginning 72 bp distal to the initiation codon. Two fragments were assembled in pKO plasmid (Stratagene) surrounding a promoterless geneticin-resistant gene containing simian virus 40 polyadenylation signals (Neo cassette) (Figure 1A). loxP sites surrounding the Neo cassette were incorporated into the vector. For

first allele targeting, G418-resistant clones were screened by genomic Southern blotting with a hybridization probe located immediately outside the 3′ homologous arm and geneticin-resistant gene coding region (Figure 1A). A clone carrying a homologous recombinant and no additional random integrants was then infected with recombinant adenovirus expressing Cre recombinase (purchased from Gene Transfer Vector Core, University of Iowa) to yield G418-sensitive clones. One of these heterozygous clones without Neo (+/− Cre) was then used for second round homologous recombination using the same targeting vector. Genomic DNA was prepared from G418-resistant clones and analyzed by PCR. Homologous recombinants identified by PCR were confirmed by Southern blot (data not shown) and several clones with both alleles of Orc2 disrupted were obtained and used for further experiments. Details of the constructs and PCR primer sequences are available upon request.

Western Blot Analysis, Immunoprecipitation, and Chromatin Fraction Preparation

Orc2 antibody was raised against a fragment of human Orc2 (27–577 amino acids) (Quintana et al., 1997). Conditions for immunoprecipitation and immunoblotting have been described (Dhar and Dutta, 2000; Thome et al., 2000). Cells were lysed in CSK buffer (100 mM NaCl and 2 mM $MgCl_2$), and the chromatin pellet extracted with DNaseI and CSK buffer (200 mM NaCl and 2 mM $MgCl_2$) to prepare the chromatin fraction (Tatsumi et al., 2000).

Cell Proliferation and Cell Cycle Analysis

Cells were counted after trypan blue treatment or by MTT based colorimetric assay (Roche). Cells were synchronized to G1-S by 0.4 mM mimosine (Sigma) for 24 hr. Standard methods were used for flow-cytometry analysis and FlowJo software used for estimation of percentage of cells in various phases of the cell cycle.

PCR Analysis for Nascent Strand Abundance

DNA replication initiation frequencies at the human c-Myc and β-globin locus were determined based on short nascent DNA abundance (Aladjem et al., 1998; Kamath and Leffak, 2001; Malott and Leffak, 1999; Tao et al., 2000). The 1–2 kb fraction of nascent DNA was isolated by alkaline gel lysis of cells in logarithmic growth and quantitated by slot blot hybridization. The results shown are based on quantitative real time PCR (ABI GeneAmp 5700) (Myc1-3, βG1-4) or competitive PCR (Myc4, βG5) to quantitate nascent strand copy number at sequence-tagged sites in the c-*myc* locus (GenBank AF176208) Myc1 (nt 3896–3964); (GenBank J00120) Myc2 (nt 1829–1891), Myc3 (nt 4488–4552), Myc4 (nt 5801–6045), and the β-globin locus (GenBank U01317) βG1 (nt 32777–32854), βG2 (nt 40916–40997), βG3 (nt 54401–54475), βG4 (nt 61821–61894), βG5 (nt 72319–72453). Competitive PCR at Myc1, Myc2, Myc3, βG2, and βG4 were used to confirm the results of the Q-PCR. Values are normalized to the abundance at Myc3. PCR primer sequences are available on request.

Assay for EBV Plasmid Replication and Maintenance

For long-term replication assay, cells were washed extensively the day after transfection and 1/10th of the cells returned to culture. Hygromycin B (Gibco-BRL) at 50–100 μg/ml was added and resistant cells selected for up to 10 days. Colonies were stained by crystal violet or used for alkaline plasmid preparation. p220.2 is identical to p201 (Yates et al., 1985) except that it contains a polylinker nearly 400 bp to the right of oriP.

Assays for the transient replication of plasmids p367 (functional EBNA-1) and p396 (deletion in the EBNA1 DNA binding domain) have been described (Yates and Camiolo, 1988). p367ΔO lacks DS of oriP (deletion, 8995–9123 of EBV). Plasmids were extracted by alkaline lysis 96 hr after transfection (Yates et al., 2000) and analyzed for presence of DpnI-resistant plasmid DNA. Plasmids without functional EBNA1 (p396) or DS of oriP (p367ΔO) did not replicate in the WT cells (data not shown), confirming that both EBNA1 and the replicator of oriP are necessary for oriP-dependent plasmid replication in the HCT116 cell line.

Details for the construction of adenovirus-expressing wild-type Orc2, pCMV-FlagΔOrc2, pSPORT-Cdt1, pEBG-geminin, and pEBG-geminin ΔDB are available on request. For the rescue of oriP expression in Δ/− cells, cells were infected with adenovirus for 1 hr, the medium changed and then transfected with p367 1 hr later. DNA was harvested at 96 hr to assess plasmid replication. To study the effect of geminin, Cdt1, or ΔOrc2, the appropriate expression plasmids were cotransfected with p367. Quantitation was done using densitometry or a phosphoimager to normalize the intensity of the DpnI-resistant fragment (replicated DNA) to that of the largest fragment created by DpnI digestion in the same lane (unreplicated DNA). These normalized intensities are presented as "relative intensities" to facilitate comparison with the first bar in each panel.

Chromatin Immunoprecipitation (ChIP)

293 cells carrying p818 were grown in Iscove's modified Dulbecco's Medium containing 275 μg/ml hygromycin B (Calbiochem). p818 (Figure 6A) is a 12.6 kb plasmid similar to p201 (Yates et al., 1985) but contains 4 kb of additional EBV DNA to the right of oriP extending to the BamHI site at 13215. 2×10^8 cells were trypsinized, washed in PBS, and fixed in PBS containing 1% para-formaldehyde for 20 min at 37°C. The fixed cells were washed, and chromatin solubilized in urea as described (Ip et al., 1988). Chromatin was sonicated to an average DNA length of 600 bp. Cross-linked chromatin with a buoyant density of 1.4 gm/ml was purified using a CsCl gradient (Orlando et al., 1997). Thirty micrograms of chromatin DNA was adjusted to 50 μl in RIPA buffer (1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5mM EDTA, 50 mM Tris HCl [pH 8.0], and 1 mM PMSF) and incubated at 4°C for 4 hr with 1.5 μg of α-EBNA-1 antibody, EBNA1.OTX1x (Chen et al., 1993), or 1 μl each of α-Orc2 or nonimmune rabbit serum. Antibody complexes were recovered on protein A Sepharose, washed 5 times with RIPA buffer (0.75 M NaCl), 2 times in LiCl buffer (0.25 M LiCl, 0.5% Triton X-100, 1% sodium deoxycholate, 1% SDS, 1 mM EDTA, and 10 mM Tris HCl [pH 8.0]), 2 times in TE (10 mM Tris HCl [pH 8.0] and 1 mM EDTA), and resuspended in 100 μl of TE. Chromatin was de-cross-linked in 0.5% SDS and proteinase K (1 mg/ml) at 37°C overnight followed by 65°C for 8 hr. DNA was extracted with phenol/chloroform and ethanol precipitated.

PCR was performed using 1/250 or 1/1250 of the ChIP DNA in 50 μl using 2.5 units Taq polymerase (Roche) for 35 cycles of 1 min at 95°C, 1 min at 60°C, and 40 s at 72°C. Four primer pairs were used to amplify regions of the plasmid simultaneously: 435 bp spanning DS at oriP (EBV 8758–9192), 334 bp located 5′ of the EBNA-1 coding region (EBV 107656–107989), 248 bp located 3′ of the EBNA-1 coding region (EBV 109726–109973), and 182 bp near the 5′ end of the hph gene (275–456 of GenBank K01193). Primer sequences are available on request. To obtain equal amplification of the four regions using control DNA from de-cross-linked chromatin, the primer pairs were used at the following concentrations: DS, 80 nM; 5′ of the EBNA-1 gene, 50 nM; 3′ of the EBNA-1 gene, 30 nM; and hph gene, 35 nM. The PCR products were separated by electrophoresis in 2% agarose gels (NuSieve 3:1, Biowhittaker), detected by staining with SYBR Green (Molecular Probes), and digitally photographed.

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Xenopus Mcm10 Binds to Origins of DNA Replication after Mcm2-7 and Stimulates Origin Binding of Cdc45

James A. Wohlschlegel,[1] Suman K. Dhar,[1] Tatyana A. Prokhorova,[2] Anindya Dutta,[1,3] and Johannes C. Walter[2,3]
[1]Department of Pathology
Brigham and Women's Hospital
75 Francis Street
[2]Department of Biological Chemistry
and Molecular Pharmacology
240 Longwood Avenue
Harvard Medical School
Boston, Massachusetts 02115

Summary

Current models suggest that the replication initiation factor Mcm10 is required for association of Mcm2-7 with origins of replication to generate the prereplicative complex (pre-RC). Here we report that *Xenopus* Mcm10 (XMcm10) is not required for origin binding of XMcm2-7. Instead, the chromatin binding of XMcm10 at the onset of DNA replication requires chromatin-bound XMcm2-7, and it is independent of Cdk2 and Cdc7. In the absence of XMcm10, XCdc45 binding, XRPA binding, and initiation-dependent plasmid supercoiling are blocked. Therefore, XMcm10 performs its function after pre-RC assembly and before origin unwinding. As one of the earliest known pre-RC activation steps, chromatin binding of XMcm10 is an attractive target for regulation by cell cycle checkpoints.

Introduction

The initiation of eukaryotic DNA replication is a highly coordinated process governed by the regulated assembly and disassembly of multiple macromolecular protein complexes (reviewed in Dutta and Bell, 1997; Kelly and Brown, 2000). Origins of replication are first recognized and bound by a six-subunit origin recognition complex (ORC). In the G_1 phase of the cell cycle, Cdc6, Cdt1, and Mcm2-7 are recruited to replication origins in an ORC-dependent process to form a prereplicative complex (pre-RC) (Maiorano et al., 2000; Nishitani et al., 2000). Current evidence suggests that the Mcm2-7 complex is the replicative helicase (reviewed in Labib and Diffley, 2001). Upon entry into S phase, DNA replication is initiated by the conversion of pre-RCs into active replication forks. This transformation requires the activity of two families of protein kinases, the Cdc7/Dbf4 kinase and the S phase cyclin-dependent kinases (Cdk), which cooperate to recruit Cdc45 to origins of DNA replication (Zou and Stillman, 2000). While there is good evidence that Cdc7 stimulates initiation by phosphorylating one or more of the Mcm2-7 complex subunits (Sclafani, 2000), the Cdk substrates are unknown. Current models suggest Cdc45 binds to the Mcm2-7 complex (Zou and Stillman, 2000), thereby promoting origin unwinding and the recruitment of replication protein A (RPA) as well as DNA polymerases to the origin (Takisawa et al., 2000; Tanaka and Nasmyth, 1998; Walter and Newport, 2000).

Xenopus egg extracts are a powerful system for studying the biochemical requirements for the initiation of DNA replication. Upon the addition of sperm chromatin to these extracts, a replication-competent nucleus is assembled around the sperm chromatin, and a single round of semiconservative DNA replication occurs (Blow and Laskey, 1986; Newport, 1987). Although replication in *Xenopus* egg extracts initiates without reference to a specific DNA sequence, the biochemical mechanism of initiation in yeast and *Xenopus* is highly conserved (Blow, 2001). Recently, nucleus-independent DNA replication has been achieved in *Xenopus* egg extracts (Walter et al., 1998). This soluble system requires the activity of two different egg extracts. First, sperm chromatin or plasmid DNA is incubated with membrane-free egg cytosol (EC). Because unfertilized *Xenopus* eggs contain a large stockpile of proteins required to undergo many rapid cell divisions, the large volume of egg cytosol contains many proteins that would normally be found in the nucleus, such as ORC, Cdc6, Cdt1, and Mcm2-7. For this reason, egg cytosol supports pre-RC formation on exogenously added DNA. However, the egg cytosol does not contain sufficient levels of Cdk and Cdc7 activities to stimulate initiation from these pre-RCs. As such, egg cytosol mimics the nuclear environment found in the G_1 phase of the cell cycle. To stimulate initiation, a concentrated nucleoplasmic extract (NPE) is added which is prepared from pseudonuclei assembled in crude egg cytoplasm. This extract supplies high levels of Cdc7 (Walter, 2000) and Cdk2 activities (J.C.W., unpublished data) and thereby stimulates initiation from previously assembled pre-RCs. NPE also contains inhibitors that block de novo binding of XMcm2-7 to chromatin. This explains why DNA must be first incubated in egg cytosol and why replication is limited to a single round in this system (Walter et al., 1998). Thus, NPE closely simulates the conditions found during the S phase of the cell cycle.

Some of the replication initiation factors identified in yeast have not yet been characterized in *Xenopus* extracts. One such factor is Mcm10. *MCM10*, also known as *DNA43*, was identified in *S. cerevisiae* in two independent genetic screens for genes required for chromosomal DNA replication and stable plasmid maintenance (Merchant et al., 1997; Solomon et al., 1992). *MCM10* mutants show a dramatic reduction in DNA replication initiation at chromosomal origins and arrest at the nonpermissive temperature with a dumbbell morphology and 2C DNA content, similar to other DNA replication initiation mutants (Merchant et al., 1997). Genetic studies have shown that mutations in *MCM10* can be suppressed by extragenic mutations in *MCM7* and *MCM5* and are synthetically lethal with mutations in *CDC45*, *ORC2*, *ORC5*, *DPB11*, *DNA2*, and genes encoding subunits of DNA polymerase ϵ and DNA polymerase δ

[3]Correspondence: johannes_walter@hms.harvard.edu (J.C.W.), adutta@rics.bwh.harvard.edu (A.D.)

(Homesley et al., 2000; Kawasaki et al., 2000; Liu et al., 2000; Merchant et al., 1997). Results from yeast two-hybrid and in vitro binding experiments suggest that Mcm10 can physically associate with the Mcm2-7 complex (Merchant et al., 1997). It was also recently demonstrated that human Mcm10 can associate with Orc2 when both proteins are overexpressed in cultured cells (Izumi et al., 2000). Mcm10 is bound to a total chromatin fraction in the G_1, S, and M phases, and it was mapped to an origin of DNA replication in asynchronous populations of cells using in vivo cross linking (Homesley et al., 2000). Finally, when yeast cells bearing a temperature sensitive allele of *MCM10* are shifted to the nonpermissive temperature after a G_1 arrest, the Mcm2-7 complex dissociates from chromatin while ORC is unaffected (Homesley et al., 2000). Together, these results have led to the model that Mcm10 is a component of the pre-RC that loads independently of ORC and that is required to mediate the association of Mcm2-7 with origins of replication.

To better understand the role of Mcm10 in DNA replication, we have cloned the *Xenopus* homolog of *MCM10* and characterized its function in *Xenopus* egg extracts. We find that immunodepletion of XMcm10 from NPE and EC results in a 5- to 10-fold decrease in DNA replication that can be rescued by the addition of recombinant XMcm10. XMcm10 does not bind chromatin in egg cytosol, but strongly associates with chromatin upon the addition of NPE in a process that requires the presence of the XMcm2-7 complex within pre-RCs and temporally precedes the chromatin loading of XCdc45 and XRPA. In extracts depleted of XMcm10, XMcm2-7 loading to form pre-RCs is unaffected while the recruitment of XCdc45 and XRPA to the chromatin is defective. Together our data argue that XMcm10 is an essential replication initiation factor that is not involved in pre-RC assembly but instead facilitates the loading of XCdc45 onto the chromatin.

Results

XMcm10 Is Enriched in Nuclear Extracts

A database search identified a *Xenopus* EST clone (db25e09) that was a potential homolog of *S. cerevisiae MCM10*. The 3.3 kb cDNA clone encoded a hypothetical open reading frame of 860 amino acids that was 10% identical and 21% homologous to *S. cerevisiae MCM10* and 56% identical and 70% homologous to human *MCM10*. This sequence is identical to the previously deposited Genbank sequence, AF314535. A fragment of the XMcm10 encoding amino acids 306–810 was purified as a His6 fusion protein and injected into rabbits. The resulting antiserum, but not the preimmune serum, specifically recognized a 100 kDa band in *Xenopus* egg cytosol (Figure 1A, lanes 1 and 2). We also compared the abundance of XMcm10 in egg cytosol and NPE, and found it to be approximately 10 times more concentrated in NPE than in egg cytosol (Figure 1B, compare lanes 1 and 4). This indicates that XMcm10 is efficiently imported into the nucleus in *Xenopus* egg extracts, in agreement with previous experiments in *S. cerevisiae*, *S. pombe*, and human cells (Izumi et al., 2000; Kawasaki et al., 2000; Merchant et al., 1997).

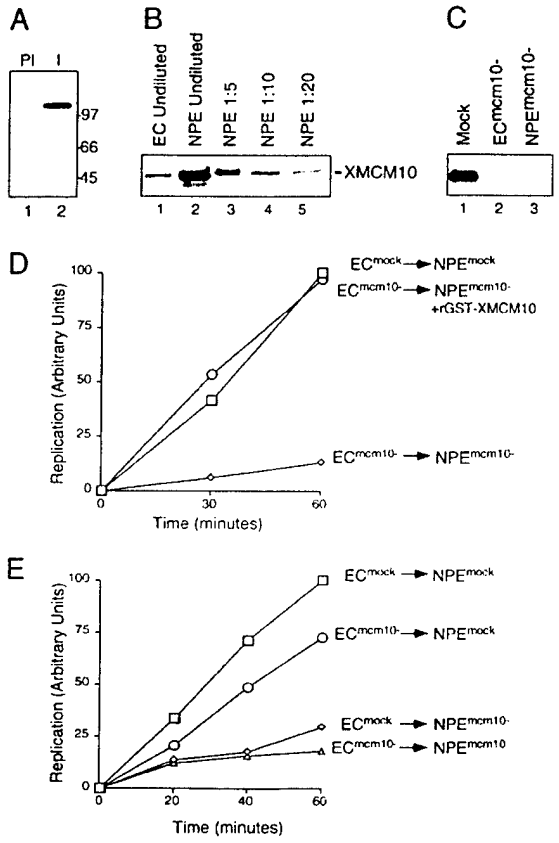

Figure 1. *Xenopus* Mcm10 Is a Nuclear Protein Required for Efficient DNA Replication

(A) Characterization of the XMcm10 antiserum. 0.5 μl of egg cytosol (EC) was immunoblotted with anti-XMcm10 antiserum (lane 2) or the corresponding preimmune serum (lane 1).

(B) XMcm10 is 10-fold enriched in NPE compared to EC. 0.5 μl EC (lane 1), 0.5 μl NPE (lane 2), 0.1 μl NPE (lane 3), 0.05 μl NPE (lane 4), and 0.025 μl NPE (lane 5) were immunoblotted with anti-XMcm10 antibody.

(C) Egg cytosol and NPE are efficiently depleted of XMcm10. 0.5 μl mock-depleted EC (lane 1), 0.5 μl XMcm10-depleted egg cytosol (lane 2), and NPE (lane 3) were immunoblotted with XMcm10 antibody.

(D) XMcm10 is essential for DNA replication. Sperm chromatin (10,000/μl final concentration) was incubated with XMcm10-depleted egg cytosol for 30 min, followed by XMcm10-depleted NPE (diamonds), or XMcm10-depleted NPE supplemented with 40 ng/μl final concentration GST-XMcm10 (circles). The relative amount of DNA replication was measured 30 and 60 min after NPE addition and compared with replication in mock-depleted extracts (squares).

(E) The XMcm10 in egg cytosol is dispensable for efficient DNA replication. Sperm chromatin was incubated with egg cytosol and replication was measured at various times following the addition of NPE. (Squares), mock-depleted EC, mock-depleted NPE; (circles) Mcm10-depleted EC, mock-depleted NPE; (diamonds), mock-depleted EC, Mcm10-depleted NPE; (triangles), Mcm10-depleted EC, Mcm10-depleted NPE.

XMcm10 Is Required for DNA Replication

To determine whether XMcm10 is required for DNA replication, we depleted it from *Xenopus* egg extracts. As shown in Figure 1C, anti-XMcm10 serum efficiently depleted XMcm10 protein from egg cytosol and NPE. Western blotting with serial dilutions of mock-depleted

extract showed that the depletion was greater than 99% (data not shown). Extracts depleted of XMcm10 exhibited a 8-fold decrease in DNA replication compared to mock-depleted extracts (Figure 1D, compare diamonds and squares), and this defect was completely reversed by addition of recombinant GST-XMcm10 (Figure 1D, circles). Therefore, the replication defect in XMcm10-depleted extracts was due to the selective removal of XMcm10. The addition of recombinant GST-XMcm10 to mock-depleted extracts had little effect on DNA replication (data not shown). These data show that XMcm10 is essential for DNA replication in *Xenopus* extracts.

We next examined whether XMcm10 must be present in egg cytosol, NPE, or both to support DNA replication (Figure 1E). Depletion of XMcm10 from the egg cytosol had only a modest effect on DNA replication (Figure 1E, circles), while depletion of XMcm10 from NPE alone led to a large decrease in DNA replication (Figure 1E, diamonds). Depletion of XMcm10 from both extracts abolished replication to a similar extent as depletion from NPE alone (Figure 1E, triangles). We showed previously that if XMcm2-7 complexes do not bind to chromatin in egg cytosol, they are unable to do so after the addition of NPE (Walter, 2000). Therefore, the lack of a requirement for XMcm10 in egg cytosol suggests that XMcm10 is not required for XMcm2-7 binding.

Kinetics of Chromatin Association of XMcm10

Since both human and budding yeast Mcm10 are tightly associated with chromatin (Homesley et al., 2000; Izumi et al., 2000; Kawasaki et al., 2000), we tested whether XMcm10 binds to chromatin during DNA replication. In contrast to XMcm7, very little XMcm10 was bound to sperm chromatin incubated in egg cytosol (Figure 2A, lane 2). However, a large increase in chromatin binding by XMcm10 occurred within 10 min after the addition of NPE (Figure 2A, lane 4). Over time, XMcm10 was displaced from the chromatin with identical kinetics as the XMcm2-7 complex (Figure 2A, lanes 4–6). When DNA replication was inhibited by the addition of the Cdk2 inhibitor $p27^{Kip}$ (as seen by the inhibition of XCdc45 and XRPA loading), XMcm10 associated with chromatin but did not dissociate over time (Figure 2A, lanes 7–9). Similarly, when DNA replication was inhibited by aphidicolin (as seen by the enhanced loading of XRPA [Walter, 2000]), XMcm10 bound to chromatin, but did not dissociate over time (Figure 2A, lanes 10–12). Thus, XMcm10 binds to chromatin before DNA replication is initiated, and it is displaced from the chromatin with the same kinetics as the XMcm2-7 complex as replication forks progress. The displacement of XMcm10 from chromatin during DNA replication is consistent with the finding in human cells that XMcm10 is more abundant on chromatin in S phase than in G_2 (Izumi et al., 2000).

That XMcm10 bound to chromatin in the presence of $p27^{Kip}$ (Figure 2A, lanes 7–9) suggested that XMcm10 might bind to chromatin before Cdc45, whose loading is dependent on Cdk activity. To test this, we performed a detailed time course of chromatin loading (Figure 2B). XMcm10 associated with chromatin less than 2 min after the addition of NPE (Figure 2B, lane 2), whereas chromatin association of XCdc45 and XRPA occurred ~6 min after the addition of NPE (Figure 2B, lane 4). Therefore, XMcm10 normally associates with chromatin before XCdc45 and XRPA.

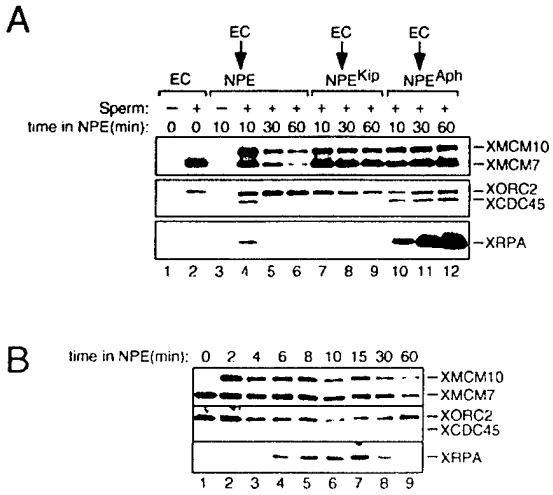

Figure 2. XMcm10 Associates with Chromatin after Pre-RC Assembly but before XCdc45 Loading

(A) Sperm chromatin was incubated with egg cytosol at 10,000/μl final concentration and isolated after 30 min (lane 2) or 10, 30, and 60 min following the addition of NPE (lanes 3–6), NPE supplemented with 1 μM GST-$p27^{Kip}$ (lanes 7–9), or NPE containing 50 μg/ml Aphidicolin (lanes 10–12). The reactions shown in lanes 1 and 3 lacked sperm chromatin. Purified chromatin was immunoblotted for XMcm10, XMcm7, Xorc2, XCdc45, and XRpa34, as indicated.

(B) Sperm chromatin was incubated with egg cytosol, and the association of XMcm10, XMcm7, Xorc2, XCdc45, and XRpa34 determined at various times after the addition of NPE.

Chromatin Association of XMcm10 Requires a Functional Pre-RC but Is Independent of Cdc7 Activity

Given the genetic and physical interaction of yeast Mcm10 with other members of the yeast Mcm2-7 complex, we tested whether the XMcm2-7 complex is required for the chromatin loading of XMcm10. We used antibodies against XMcm7 to deplete the XMcm2-7 complex from egg cytosol and then examined the chromatin association of XMcm10 after addition of NPE. It was not necessary to deplete XMcm7 from NPE, because de novo binding of XMcm2-7 does not occur after addition of NPE (Walter et al., 1998; Walter, 2000). XMcm2-7-depleted extracts were unable to recruit XMcm10 to the chromatin (Figure 3A, compare lanes 3 and 4). Similarly, the addition to egg cytosol of geminin, which blocks XMcm2-7 loading through its association with Cdt1 (Tada et al., 2001; Wohlschlegel et al., 2000), also prevented the recruitment of XMcm10 to replication origins (Figure 3B). Together, these data suggest that the presence on chromatin of the XMcm2-7 complex is required for the recruitment of XMcm10 to replication origins.

We next determined whether XCdc7 was required for chromatin binding of XMcm10. We find that XMcm10 was efficiently loaded onto chromatin in the absence of XCdc7 (Figure 3C, lane 4). The effectiveness of the XCdc7-depletion was demonstrated by the absence of XRPA chromatin binding in the depleted extracts (Figure

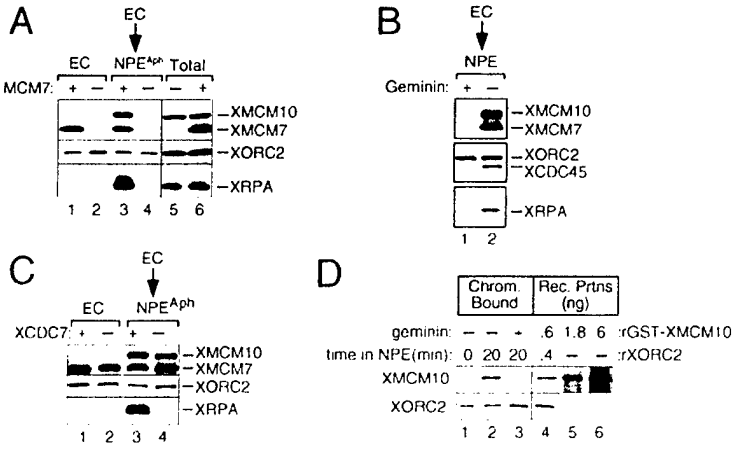

Figure 3. The Association of XMcm10 with Chromatin Requires XMcm2-7 but Is Independent of XCdc7

(A) XMcm10 does not bind to chromatin in XMcm7-depleted extracts. Sperm chromatin was incubated with mock-depleted (lanes 1 and 3) or XMcm7-depleted egg cytosol (lanes 2 and 4). In lanes 1 and 2, chromatin was isolated after incubation in egg cytosol, whereas in lanes 3 and 4, it was isolated after a further 20 min incubation in NPE containing aphidicolin. Chromatin and 0.5 μl aliquots of mock-depleted (lane 6) and XMcm7-depleted egg cytosol (lane 5) were immunoblotted for XMcm10, XMcm7, XOrc2, and XRpa34, as indicated.

(B) Geminin inhibits chromatin binding of XMcm10. Sperm chromatin was incubated with egg cytosol containing 150 nM human geminin (lane 1) or buffer (lane 2), and isolated after a further 10 min incubation with NPE.

(C) Chromatin association of XMcm10 does not require XCdc7. Sperm chromatin was incubated with mock-depleted (lanes 1 and 3) or XCdc7-depleted egg cytosol (lanes 2 and 4). After 30 min, chromatin was isolated (lanes 1 and 2) or incubated for a further 30 min with mock-depleted or XCdc7-depleted NPE containing aphidicolin and then isolated (lanes 3 and 4).

(D) The ratio of XMcm10 to XOrc2 on chromatin is ~2:1. Sperm chromatin was isolated after incubation in egg cytosol (lane 1), egg cytosol followed by a 20 min incubation in NPE containing aphidicolin (lane 2), or egg cytosol containing 150 nM geminin followed by a 20 min incubation in NPE containing aphidicolin (lane 3). Chromatin-bound proteins from 10,000 sperm were immunoblotted alongside known quantities of purified GST-XMcm10 or his-XOrc2 (lanes 4–6). Lane 4 contains equimolar amounts of XMcm10 and XOrc2.

3C, lane 4). Figure 2A showed that the addition of the Cdk2 inhibitor $p27^{Kip}$ did not inhibit chromatin binding by XMcm10. Therefore, recruitment of XMcm10 to replication origins is independent of both Cdk2 and Cdc7, but requires the XMcm2-7 complex.

It was important to determine how much XMcm10 loads onto origins at the onset of DNA replication. To this end, sperm chromatin was incubated in egg cytosol followed by NPE containing aphidicolin. Chromatin was then isolated and analyzed on a Western blot alongside known quantities of purified GST-XMcm10. We found that ~1 ng of XMcm10 is loaded onto 10,000 sperm (Figure 3D, compare lane 2 with lanes 4 and 5). Since each sperm contains 2.9×10^9 basepairs of DNA, XMcm10 binds on average once every 5000 basepairs. In the same experiment, ~0.3 ng XOrc2 was found to bind to 10,000 sperm, or one molecule per 11,000 basepairs (Figure 3D, compare lanes 1 and 4), which is similar to previous reports (Rowles et al., 1996; Walter and Newport, 1997) and close to the experimentally determined replicon size of ~10 kb (Blow et al., 2001; Hyrien and Mechali, 1993; Mahbubani et al., 1992; Walter and Newport, 1997). Given that all the XMcm10 loaded in this experiment was geminin sensitive (Figure 3D, lane 3), we conclude that roughly two molecules of XMcm10 bind per origin of DNA replication at the onset of DNA replication. The concentration of XMcm10 in *Xenopus* egg cytosol is 16 ng/μl (data not shown).

XMcm10 Is Required for Chromatin Binding of XCdc45 and Origin Unwinding, but Not for XMcm2-7 Complex Recruitment

Experiments in yeast strongly suggested that Mcm10 is required for the initiation of DNA replication (Merchant et al., 1997). To determine at what step of replication initiation XMcm10 performs its function, we carried out chromatin binding experiments in XMcm10-depleted extracts (Figure 4A). Sperm chromatin was incubated in XMcm10-depleted egg cytosol, followed by the addition of XMcm10-depleted NPE containing aphidicolin. The aphidicolin served to arrest the system after initiation had occurred. Under these conditions, normal levels of XMcm7 bound to chromatin (Figure 4A, compare lanes 1 and 2). Sperm isolated from XMcm10-depleted egg cytosol before addition of NPE also contained the same amount of XMcm7 and XMcm3 as mock-depleted extracts (data not shown). Strikingly, in the absence of XMcm10, XCdc45 and XRPA binding in NPE was severely reduced (Figure 4A, compare lanes 1 and 2), and the reduced binding was reversed by the addition of recombinant XMcm10 (Figure 4A, lane 3). In this experiment, depletion of XMcm10 caused an ~6-fold reduction in DNA replication that was fully rescued by GST-XMcm10, and the overall efficiency of DNA replication was ~100% (Figure 4B, see legend). Similar defects in XRPA and XCdc45 binding were observed in extracts lacking aphidicolin (data not shown). We also examined the binding of XCdc7 to chromatin that normally occurs after NPE addition (Walter, 2000) in XMcm10-depleted extracts. We found a small but reproducible decrease in the amount of chromatin-bound XCdc7 in XMcm10-depleted extracts (Figure 4C). Thus, although our data show that XCdc7 loads onto chromatin independently of XMcm10, we cannot rule out the possibility that there is a pool of XCdc7 whose chromatin association is XMcm10 dependent. Together, these experiments show that XMcm10 is not required for prereplicative complex assembly, but rather for the recruitment of XCdc45 and RPA to origins of replication.

The lack of XCdc45 and XRPA binding in XMcm10-depleted extracts suggests that XMcm10 is required for origin unwinding (Walter and Newport, 2000). To test this directly, a circular plasmid was incubated in egg cytosol followed by NPE containing aphidicolin, and its

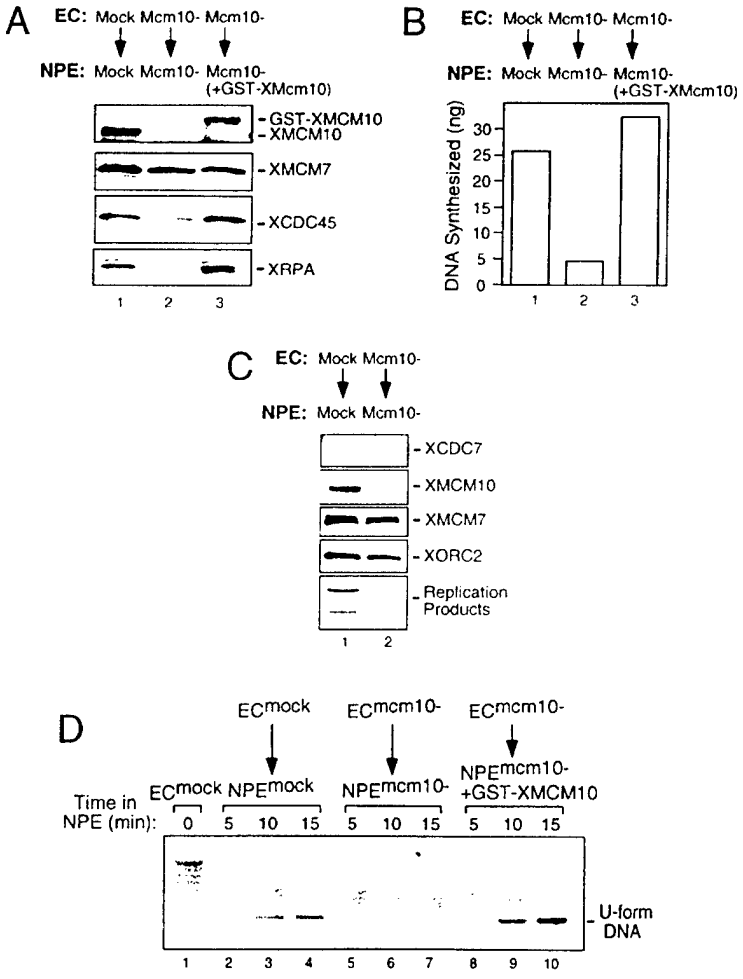

Figure 4. XMcm10 Is Required for the Chromatin Association of XCdc45 and Origin Unwinding

(A) Sperm chromatin was incubated with mock-depleted egg cytosol followed by mock-depleted NPE (lane 1) or XMcm10-depleted egg cytosol followed by XMcm10-depleted NPE (lanes 2 and 3). In lane 3, the NPE contained 40 ng/μl GST-XMcm10, and NPE in all three reactions was supplemented with 50 ng/μl human cyclin A/Cdk2 (see Experimental Procedures) and aphidicolin. Chromatin association of XMcm7, XCdc45, and XRPA was determined by immunoblotting after 30 min incubation in NPE.

(B) The same reactions as in (A), but lacking aphidicolin, were supplemented with [α-^{32}P]dATP, and replication from 8,300 sperm was measured 30 min after NPE addition. Because the amount of input DNA (27 ng) matched the amount of newly synthesized DNA (see Figure 4B), replication efficiency was ~100%.

(C) Sperm chromatin was incubated with mock-depleted egg cytosol followed by mock-depleted NPE (lane 1) or XMcm10-depleted egg cytosol followed by XMcm10-depleted NPE (lane 2). Both mock and XMcm10-depleted NPE were treated with p27^{KIP} to arrest replication initiation after XCdc7 loading (Walter, 2000). Chromatin association of XMcm7, XOrc2, XCdc7, and XMcm10 was determined by immunoblotting after 20 min incubation in NPE. DNA replication was also assessed in mock and XMcm10-depleted extracts after 30 min incubation in NPE.

(D) Origin unwinding does not occur in XMcm10-depleted extracts. pBS (40 ng/μl final concentration) was incubated with mock-depleted (lanes 1–4) or Mcm10-depleted egg cytosol for 30 min (lanes 5–10). DNA was then isolated (lane 1) or further incubated after addition of mock-depleted NPE (lanes 2–4), XMcm10-depleted NPE (lanes 5–7), or XMcm10-depleted NPE containing 40 ng/μl GST-XMcm10 (lanes 8–10), and then isolated. Origin unwinding was monitored by the appearance of negatively supercoiled "U" form DNA on a chloroquine agarose gel.

topology was analyzed on an agarose gel containing chloroquine. Origin unwinding occurs after NPE addition and can be separated into two steps (Walter and Newport, 2000). The first step requires Mcm2-7, Cdk2, Cdc7, and Cdc45 but is RPA-independent and involves a significant but limited supercoiling of the plasmid (see Figures 3A–3F in Walter and Newport, 2000). The second step requires a single-stranded DNA binding protein (RPA or *E. Coli* SSB) and results in a highly unwound species (U form DNA). The amount of U form DNA generated in XMcm10-depleted extracts was severely reduced compared to mock-depleted extracts (Figure 4D, lanes 2–4 versus 5–7), and this defect was rescued by the addition of recombinant XMcm10 (Figure 4D, lane 8–10). Moreover, in the absence of XMcm10, there was no partial supercoiling of the plasmid. The small change in linking number that is observed upon addition of NPE (Figure 4D, compare lanes 1 and 5) is ORC-independent (Walter et al., 1998) and therefore not related to initiation of DNA replication. Therefore, using the DNA topology assay, we find that origin unwinding is blocked at an early step in XMcm10-depleted extracts. The results of the chromatin binding and plasmid supercoiling assays therefore both show a requirement for XMcm10 at an early stage of origin unwinding.

Discussion

In this paper, we report the cloning and characterization of the *Xenopus* homolog of the replication factor *MCM10*. Using the *Xenopus* cell-free DNA replication system, we show that XMcm10 is required for DNA replication and that it is recruited to origins before XCdc45 and XRPA. XMcm10 recruitment is dependent on the XMcm2-7 complex, but it is independent of Cdk2 and Cdc7 activities. We also show that XMcm10 is required for the recruitment of XCdc45 and XRPA to replication origins, and as such, its depletion from extracts leads to a defect in origin unwinding. Based on these findings, we present a model for XMcm10 function in which XMcm10 is not required for pre-RC assembly but instead participates in the activation of pre-RCs at the onset of S phase to facilitate their conversion to active replication forks (Figure 5).

MCM10 was initially identified in yeast as a gene re-

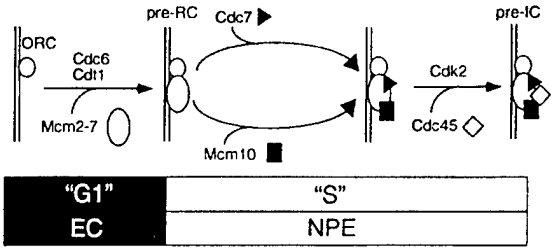

Figure 5. Model for the Assembly of Replication Factors at Origins of Replication in *Xenopus* Extracts

quired for chromosomal DNA replication and plasmid stability (Merchant et al., 1997; Solomon et al., 1992). Biochemical and genetic studies have since given rise to the current model for Mcm10 function in which Mcm10 is a critical component of the pre-RC that cooperates with ORC to recruit the Mcm2-7 complex to replication origins (Homesley et al., 2000; Izumi et al., 2000; Kawasaki et al., 2000; Merchant et al., 1997). In contrast, we have provided three lines of evidence that the *Xenopus* homolog of Mcm10 is not required for the recruitment or the maintenance of the XMcm2-7 complex on chromatin. First, DNA replication in *Xenopus* extracts does not requires XMcm10 to be present in egg cytosol during the formation of pre-RCs (Figure 1E). Importantly, this incubation in egg cytosol represents the only window of opportunity for pre-RC assembly in this system. Second, we show that virtually no XMcm10 associates with chromatin in egg cytosol during pre-RC assembly, whereas one to two XMcm10 molecules bind to origins upon the addition of NPE (Figures 2A and 3D). Finally, in XMcm10-depleted extracts, there is no reduction in the efficiency of XMcm2-7 loading onto chromatin (Figure 4A). Differences in our studies and the yeast studies reporting the requirement of Mcm10 for the stable association of Mcm2-7 with chromatin may result from the fact that those studies assayed the presence of Mcm2-7 in the general chromatin fraction and not just at origins. Alternatively, discrepancies with the yeast data may reflect genuine differences between *X. laevis* and *S. cerevisiae* with regard to the order of events during replication initiation.

Although XMcm10 is not required for the stable chromatin loading of the Mcm2-7 complex, we find that the converse is true. The recruitment of XMcm10 to origins requires the prior chromatin association of the XMcm2-7 complex (Figures 3A and 3B). This finding is consistent with the strong genetic and physical interactions between Mcm10 and the Mcm2-7 complex (Homesley et al., 2000; Merchant et al., 1997) and also provides the molecular basis for the localization of XMcm10 to replication origins. These observations raise the question whether XMcm10 should be considered a component of the prereplication complex, which was originally defined as the protein complex that assembles at origins of DNA replication in the G_1 phase of the cell cycle in budding yeast (Diffley et al., 1994). Since egg cytosol and NPE recapitulate key features of the G_1 and S phases of the cell cycle, respectively (see Introduction), and because XMcm10 loads onto origins only after NPE addition, we suggest that chromatin binding of XMcm10 is an S phase event. This interpretation is supported by the recent observation by Izumi and colleagues that the human Mcm10 binds to chromatin at the onset of S phase in HeLa cells (Izumi et al., 2001). It still remains unclear what triggers Mcm10 binding to chromatin. Our data suggest that Cdk activity is not required for this event. Therefore, it may be that Mcm10 binds to pre-RCs when a sufficient concentration of this protein is present in cells. This model is consistent with our finding that Mcm10 binds to chromatin only in NPE, where it is enriched, and that it binds soon after its expression level increases in Hela cells (Izumi et al., 2001).

At the onset of S phase, pre-RCs are converted into initiation complexes by the Cdk- and Cdc7-dependent chromatin loading of Cdc45 onto replication origins (Jares and Blow, 2000; Takisawa et al., 2000; Walter, 2000; Zou and Stillman, 2000). Our results show that the recruitment of XCdc45 and XRPA to replication origins also requires XMcm10 (Figure 4A). The involvement of Mcm10 in this process provides an explanation for the synthetic lethality previously seen between mutant alleles of *CDC45* and *MCM10* (Homesley et al., 2000). Interestingly, the two mutant alleles of the Mcm2-7 complex that were shown to specifically suppress the *mcm10-1* mutant were initially identified as suppressors of *cdc45-1*, further emphasizing the functional relationship between Cdc45 and Mcm10 (Homesley et al., 2000). Thus, our findings that XMcm10 is involved in the recruitment of XCdc45 to replication origins in an XMcm2-7-dependent fashion is consistent with previous genetic studies. Although the exact mechanism by which Mcm10 functions to recruit Cdc45 is unclear, it is intriguing that Mcm10 is recruited to replication origins independently of both Cdc7 and Cdk2 activity, yet all three are required for Cdc45 loading. It is tempting to speculate that the phosphorylation of Mcm10 by Cdc7 and/or Cdk2 may be a critical step in the recruitment of Cdc45 to origins of replication.

Our results suggest that the chromatin association of Mcm10 is the earliest detectable step in the activation of pre-RCs at the onset of DNA replication, as it occurs before Cdc45 loading and independently of Cdc7 and Cdk2. This finding raises the possibility that Mcm10 may play an important role in determining which origins fire and the time in S phase at which they fire. Previous studies in budding yeast have identified the chromatin association of Cdc45 as an important marker for the timing of origin activation and also suggested that the S phase checkpoint may regulate the chromatin association of Cdc45 through the action of the checkpoint kinase Rad53 (Aparicio et al., 1999; Costanzo et al., 2000; Zou and Stillman, 2000). Our data raise the possibility that the regulated chromatin association of Cdc45 seen in those studies could also be affected by the regulated chromatin association of Mcm10. Evidence for involvement of Mcm10 in the DNA replication checkpoint comes from two different observations. One is that *MCM10* mutants are synthetically lethal with *RAD53* mutants (Kawasaki et al., 2000). Second, inactivation of Mcm10 in yeast cells during S phase leads to a loss of chromosome integrity that could result from the lack of an intact S phase checkpoint (Kawasaki et al., 2000).

Although our results clearly indicate a role for Mcm10

in initiation complex formation, they do not rule out an additional role for Mcm10 in elongation. Our finding that XMcm10 is displaced from the chromatin with identical kinetics to the XMcm2-7 complex is suggestive of a role in elongation. A recent study in *S. cerevisiae* showed that Mcm10 is required for the completion of S phase after release from a hydroxyurea block (Kawasaki et al., 2000). Since no new initiation events are required to complete S phase under these conditions, these results suggest that Mcm10 is also required for the elongation stage of DNA replication. The genetic interactions of *MCM10* with subunits of Polϵ, Polδ, and DNA2, proteins known to be involved in elongation, also support a role for Mcm10 in elongation (Kawasaki et al., 2000; Liu et al., 2000). Could the role of Mcm10 in elongation be similar to its role in initiation? Studies of a *cdc45* degron mutant showed that newly synthesized Cdc45 could be functionally reincorporated into stalled replication forks during hydroxyurea arrest and then subsequently perform its essential role in elongation (Tercero et al., 2000). As this recruitment of Cdc45 to replication forks is thought to be independent of pre-RCs, it is tempting to speculate that this recruitment of Cdc45 to stalled replication forks may require Mcm10. Thus, it may be possible that Mcm10 is not only required for the recruitment of Cdc45 to the replication origins during the initiation of DNA replication but also cooperates with Cdc45 during elongation and is required for its stable association with replication forks.

Experimental Procedures

Cloning of XMcm10 and Plasmid Construction

The cDNA encoding XMcm10 (dbEST Id: 4548867) was identified by searching the *Xenopus* EST database for genes with homology to *S. cerevisiae MCM10* and obtained from the Washington University Genome Sequencing Center. We have sequenced the entire cDNA and find that it is identical to a previsouly deposited sequence in Genbank (AF314535). The entire open reading encoding amino acids 1–860 was PCR amplified using primers containing a BglII site in the 5′ primer and a SalI site in the 3′ primer. The PCR product was digested with BglII and SalI and ligated to a variant of pGEX-2T digested with BamHI and XhoI. We also cloned a fragment of XMcm10 encoding amino acids 306–810 from IMAGE clone PBX0047E07 into pET28a for expression as a His-tagged fusion protein. Briefly, the plasmid was digested with BamHI and NotI, and the resulting 1.6 kb fragment was ligated to pET28a (Novagen, Madison, WI) that had been digested with the same enzymes.

Production of Antibodies against XMcm10

pET28a-XMcm10 (amino acids 306–810) was transformed into bacterial strain BL21(DE3), and expression of the His-tagged fusion protein was induced by the addition of IPTG to a final concentration of 1 mM. The protein was then solubilized in 8 M urea and purified over a Nickel-NTA agarose column as described by the manufacturer (Qiagen, Valencia, CA). Rabbits were immunized with this antigen (Cocalico Biologicals, Reamstown, PA), and the resulting antiserum reacted specifically with His6-XMcm10 (amino acids 306–810).

Purification of GST-Mcm10

pGEX-XMcm10 was expressed in BL21(DE3) cells and purified using GSH-agarose according to the manufacturer's instructions (Pharmacia Biotech, Piscataway, NJ). The purified protein was then dialyzed against egg lysis buffer (ELB; 250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM HEPES [pH 7.7]), and its concentration was estimated using the Biorad protein assay.

Immunological Methods

XMcm10 was depleted from 1 volume of egg cytosol or NPE by three sequential incubations of 2, 2, and 12 hr with 0.2 volumes of protein A Sepharose fast flow (Pharmacia) that had been prebound to 0.6 volumes of XMcm10 antiserum or the corresponding preimmune serum. Depletions of XCdc7 and XMcm7 were described previously (Walter, 2000). Western blotting with XMcm10 antiserum was performed by using antiserum diluted to 1:5000. Western blotting with antibodies against XMcm7, XCdc7, XOrc2, XCdc45, and XRPA were described previously (Walter and Newport, 2000; Walter et al., 1998; Walter, 2000).

DNA Replication, DNA Unwinding, and Chromatin Binding Assays

Extract preparation and replication assays were carried out as described (Walter et al., 1998). Chromatin binding assays were performed as described in Walter (2000). DNA unwinding assays were performed according to Walter and Newport (2000). The lengthy immunodepletion procedure required to remove XMcm10 (see above) often led to significant nonspecific inactivation of NPE through loss of Cdk activity. Therefore, in the experiments presented in Figures 4A and 4B, NPE was supplemented with 50 ng/μl final concentration cyclin A/Cdk2 (Wohlschlegel et al., 2001). The amount of DNA synthesized in Figure 4B was calculated as described (Blow and Laskey, 1986) assuming 50 μM endogenous pools of dNTPs.

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